PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C07K 14/705, C12N 5/06, 5/08, 5/10, 15/12, A61K 38/17, G01N 33/68, A61P 35/00

A1

(11) International Publication Number: **WO 00/24778**

(43) International Publication Date: 4 May 2000 (04.05.00)

(21) International Application Number: PCT/US99/24887

(22) International Filing Date: 22 October 1999 (22.10.99)

(30) Priority Data:
60/105,577 26 October 1998 (26.10.98) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 60/105,577 (CIP)
Filed on 26 October 1998 (26.10.98)

(71) Applicant *(for all designated States except US)*: THE GOVERNMENT OF THE UNITED STATES OF AMERICA represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Office of Technology Transfer, National Institutes of Health, Suite 325, 6011 Executive Boulevard, Rockville, MD 20852 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: PARKHURST, Maria, R. [US/US]; 4938 Grace Court, Ellicott City, MD 21403 (US). ROSENBERG, Steven, A. [US/US]; 10104 Inn Gate Road, Potomac, MD 20854 (US). KAWAKAMI, Yutaka [JP/JP]; 757–120 Katakuracho, Kanagawa–ku, Yokohama–shi, Kanagawa–ken 221–0861 (JP). ROBBINS, Paul, F. [US/US]; 2 Wandering Trail Court, Potomac, MD 20854 (US).

(74) Agents: FEILER, William, S. et al.; Morgan & Finnegan, L.L.P., 345 Park Avenue, New York, NY 10154 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: HLA–A2 AND HLA–DR SPECIFIC PEPTIDE EPITOPES FROM THE MELANOMA ANTIGEN TRP2

(57) Abstract

Novel HLA–Class I and HLA Class II restricted epitopes of the melanoma antigen tyrosinase–related protein 2 are described. A novel HLA–A*0201 restricted epitopes present in the melanoma antigen tyrosinase–related protein 2 (TRP2) is disclosed. The invention relates to a nine–amino acid peptide designated TRP2 (180–188). The peptide is demonstrated to induce cytotoxic T lymphocytes (CTL) which specifically react with, and lyse, melanoma cells in the context of HLA–A2 or HLA–A*0201. The invention further relates to a nine–amino acid peptide derived from TRP2 which induces helper T lymphocytes in the context of HLA–DR. The invention also relates to diagnostic methods and pharmaceutical compositions which employ the tumor antigen therapeutically and prophylactically.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

TITLE OF THE INVENTION

HLA-A2 AND HLA-DR SPECIFIC PEPTIDE EPITOPES FROM THE MELANOMA ANTIGEN TRP2

FIELD OF THE INVENTION

The present invention relates to the area of cancer diagnostics and therapeutics. More specifically, the invention relates to the identification of novel HLA-A2 and HLA-DR specific epitopes from the melanoma antigen tyrosinase-related protein 2 (TRP2) and their use in diagnostic methods. The invention further relates to pharmaceutical compositions which employ these peptides, therapeutically and prophylactically.

BACKGROUND OF THE INVENTION

Clinical evidence strongly suggests that T lymphocytes can mediate the regression of melanoma. In one study, the adoptive transfer of autologous tumor infiltrating lymphocytes (TIL) with IL-2 significantly reduced tumor burden in 30-40% of patients with metastatic disease (1, 2). Some populations of tumor reactive cytotoxic T lymphocytes (CTL) derived from TIL or from lymphocytes stimulated *in vitro* with tumor cells recognize non-mutated proteins expressed on melanomas from multiple patients. At least 14 normal self proteins have been identified as melanoma antigens (3-5), and results from several clinical investigations suggest that class I MHC restricted recognition of epitopes from such proteins by CTL may be involved in tumor regression (see, for example, 6-9).

One such self protein is tyrosinase-related protein 2 or TRP2 (see 10, 11 and WO 97/29195). TRP2 has been identified as a melanoma antigen recognized by tumor reactive CTL in both the mouse (12) and human (13). In the murine model, several B16 reactive CTL lines generated from splenocytes of C57BL/6 mice immunized with irradiated B16 melanoma cells recognized a TRP2(181-188) (VYDFFVWL: SEQ ID NO: 4) antigen in the context of H-2K^b. In addition, a T cell line raised by repeated *in vitro* stimulation of murine splenocytes with the TRP2 (181-188) peptide recognized B16 melanoma and eliminated 3 day old established

pulmonary micrometastases *in vivo* (12). In the human, the TRP2 (197-205) (LLGPGRPYR: SEQ ID NO: 56) peptide was identified as the HLA-A31 restricted epitope recognized by a CTL clone derived from a population of TIL (TIL586). The adoptive transfer of this TIL with IL-2 into the autologous patient resulted in an objective clinical response (13). The same peptide was also recognized by an independent TIL (TIL1244) in the context of HLA-A33 (14).

However, HLA-A31 and HLA-A33 were only expressed in 6% and 2% respectively of the 412 melanoma patients referred to the National Cancer Institute (15). By comparison, HLA-A2 is expressed in about 47% of melanoma patients in the United States with the most common subtype HLA-A*0201 expressed in approximately 98% of the HLA-A2 positive patients in North America (16). In addition, since the expression of non-mutated melanoma antigens is heterogeneous among tumors isolated from different patients and between individual cells from single lesions, the development of immunotherapies based on as many antigens as possible may be clinically beneficial. Therefore, to increase the number of patients eligible for TRP2 based treatments, it is desirable to identify additional HLA-A2 restricted epitopes from the TRP2 protein.

A number of antigens that are recognized by human class II restricted, melanoma reactive T cells have now been described. Initial studies demonstrated that $CD4^+$ T cell clones raised by the stimulation of PBL, as well as $CD4^+$ tumor infiltrating lymphocytes (TIL) could recognize autologous melanoma cells (Chen, Q. and Hershey, P. 1992) (Radrizzani, M. et al 1991). Additional data indicated that $CD4^+$ T cells could recognize shared melanoma antigens (Topalian et al, Int. J. Cancer, 1994). Subsequently, the widely shared melanosomal antigen tyrosinase was found to be recognized by HLA-DRB1*0401 reactive T cells and 2 peptide epitopes from this molecule were identified (Topalian et al, J. Exp. Med. 1996). The cancer-testis antigen MAGE-3, a shared melanoma antigen identified initially through the use of class I restricted T cells, has also been shown to be recognized by class II restricted T cells. In one study, *in vitro* sensitization carried out with the recombinant MAGE-3 protein was shown to elicit T cells that recognized peptide epitopes from this antigen

in the context of the class II HLA-DRB1*1301 and 1302 alleles (Chaux et al, 1999, J. Exp. Med.).

Additional studies have resulted in the identification of unique antigens recognized by class II restricted, melanoma reactive T cells. Using a biochemical approach, a mutated triosephosphate isomerase epitope was found to be recognized by HLA-DRB1*0101 restricted, melanoma reactive T cells (Pieper et al, J. Exp. Med. 1999). A novel product generated by a chromomosomal rearrangement resulted in the juxtapositon of sequences from the low density lipid receptor and the 2-a-L-fucosyltransferase gene, and a T cell epitope that was recognized in the context of HLA-DRB1*0101 was identified (Wang et al, J. Exp. Med. 1999). In addition, a mutated product of the CDC27 gene was found to be recognized by HLADRB1*0401 restricted, melanoma reactive T cells (Wang et al, Science, 1999).

It is therefore desirable to identify other novel HLA-Class II restricted cancer associated peptide epitopes for use in immunotherapy.

SUMMARY OF THE INVENTION

The invention relates to the identification of HLA-Class I and -Class II restricted epitopes present in tyrosinase-related protein 2, and analogs of the epitopes.

The invention relates to the identification of HLA-A2 restricted epitopes present in the 519 amino acid melanoma antigen known as tyrosinase-related protein 2 (TRP2). In particular, the invention relates to a nine-amino acid peptide designated TRP2 (180-188) which has the amino acid sequence SVYDFFVWL and is demonstrated to induce cytotoxic T lymphocytes (CTL) which specifically react with, and lyse, melanoma cells in the context of HLA-A*0201.

The invention further relates to analogs of TRP2 (180-188) which are capable of specifically reacting with, and lysing, melanoma cells in the context of HLA-A*0201.

The invention therefore also relates to nucleic acid sequences which encode TRP2 (180-188) and analogs thereof.

The invention further relates to a diagnostic method which utilizes TRP2 (180-188) and/or analogs thereof to detect melanoma in a mammal.

The invention also relates to pharmaceutical compositions which comprise TRP2 (180-188), and/or analogs thereof, either alone or in combination with other HLA-A2 specific peptides encoded by TRP2 or other melanoma antigens, and the use of these compositions in the prevention or treatment of melanoma in a mammal.

The invention further relates to pharmaceutical compositions which comprise nucleic acid molecules encoding TRP2 (180-188), and/or analogs thereof, either alone or in combination with nucleic acid sequences encoding other HLA-A2 specific melanoma antigen peptides and the use of these compositions in the prevention or treatment of melanoma in a mammal.

The invention therefore also relates to methods of producing a TRP2 (180-188)-specific T cell response in a mammal utilizing the compositions of the invention.

The present invention further relates to isolated T cells having specific reactivity to the TRP2 (180-188) peptide or analogs thereof, to methods of preparing such T-cells, to the use of such T cells as diagnostics and therapeutic reagents, and to pharmaceutical compositions comprising the T cells.

The invention also relates to target cells, preferably dendritic cells, which have been incubated in vitro with the TRP2 (180-188) peptide and/or analogs thereof, to the use of such target cells as diagnostic and therapeutic reagents, and to pharmaceutical compositions which comprise the target cells.

The invention relates to TRP2 peptides having at least 9 amino acids and derived from TRP2 which comprise the amino acid sequence Xaa_1 LPYWNFAT Xaa_2, wherein Xaa_1 is any one of 20 naturally occurring amino acids, preferably an amino acid selected from the group consisting of Ala, Gln, Val, Ser, Leu, Ile or no amino acid and Xaa_2 is any one of 20 naturally occurring amino acids, preferably Gly, or no amino acid, and analogs thereof which are recognized by immune cells in the context of HLA Class II.

The invention further relates to nucleic acid sequences which encode a TRP2 peptide comprising at least 9 amino acids comprising the amino acid sequence Xaa_1 LPYWNFAT Xaa_2 and analogs thereof, wherein Xaa_1 any one of 20 naturally occurring amino acids, preferably is an amino acid selected from the group consisting of Ala, Gln, Val, Ser, Leu, Ile or no amino acid and Xaa_2 is any one of 20 naturally occurring amino acids, preferably Gly, or no amino acid, and analogs thereof which are recognized by immune cells in the context of HLA Class II.

Another aspect of the invention is a vector comprising nucleic acid sequences which encode a TRP2 peptide comprising at least 9 amino acids and comprising the amino acid sequence Xaa_1 LPYWNFATXaa$_2$, and analogs thereof, wherein Xaa_1 any one of 20 naturally occurring amino acids, preferably is an amino acid selected from the group consisting of Ala, Gln, Val, Ser, Leu, Ile or no amino acid and Xaa_2 is any one of 20 naturally occurring amino acids, preferably Gly, or no amino acid, and analogs thereof which are recognized by immune cells in the context of HLA Class II.

Yet another aspect of the invention are host cells transformed or transfected by the vector encoding a TRP2 peptide and optionally are transformed or transfected with an HLA-Class II molecule and the use of the host cells as immunogens or vaccines against cancer.

The invention further provides a diagnostic kit and a diagnostic method which utilizes the TRP2 peptides having at least 9 amino acids comprising the amino acid sequence Xaa_1 LPYWNFATXaa$_2$ wherein Xaa_1 any one of 20 naturally occurring amino acids, preferably is an amino acid selected from the group consisting of Ala, Gln, Val, Ser, Leu, Ile or no amino acid and Xaa_2 is any one of 20 naturally occurring amino acids, preferably Gly, or no amino acid, and analogs thereof which are recognized by immune cells in the context of HLA Class II or analogs thereof, or the nucleic acid sequence encoding same, or complementary nucleic acid sequence to detect melanoma in a mammal.

Another aspect of the invention is a pharmaceutical composition comprising a TRP2 peptide having at least 9 amino acids and comprising the amino

acid $Xaa_1LPYWNFATXaa_2$, wherein Xaa_1 is any one of 20 naturally occurring amino acids, preferably an amino acid selected from the group consisting of Ala, Gln, Val, Ser, Leu, Ile or no amino acid and Xaa_2 is any one of 20 naturally occurring amino acids, preferably Gly, or no amino acid, and analogs thereof which are recognized by immune cells in the context of HLA Class II, or analogs thereof, or combinations thereof, either alone or in combination with other HLA-Class II specific peptides encoded by TRP 2 or other melanoma antigens for use as an immunogen for eliciting specific helper T cell immune responses and as a vaccine in the prevention or treatment of melanoma in a mammal.

The invention further relates to pharmaceutical compositions which comprise nucleic acid sequences encoding a TRP2 peptide comprising at least 9 amino acids and comprising the amino acid sequence $Xaa_1LPYWNFATXaa_2$, wherein Xaa_1 is any one of 20 naturally occurring amino acids, preferably an amino acid selected from the group consisting of Ala, Gln, Val, Ser, Leu, Ile or no amino acid and Xaa_2 is any one of 20 naturally occurring amino acids, preferably Gly, or no amino acid, and analogs thereof which are recognized by immune cells in the context of HLA Class II, or combinations thereof, alone or in combination with nucleic acid sequences encoding other HLA Class II-specific melanoma antigen peptides for use in the prevention or treatment of melanoma in a mammal.

The invention further relates to methods of producing an HLA-Class II restricted T cell response to a TRP2 peptide having at least 9 amino acids and comprising the amino acid sequence $Xaa_1LPYWNFATXaa_2$ wherein Xaa_1 is any one of 20 naturally occurring amino acids, preferably an amino acid selected from the group consisting of Ala, Gln, Val, Ser, Leu, Ile or no amino acid and Xaa_2 is any one of 20 naturally occurring amino acids, preferably Gly, or no amino acid, and analogs thereof which are recognized by immune cells in the context of HLA Class II, in a mammal utilizing the compositions of the present invention.

The present invention further relates to isolated T cells having specific reactivity to a TRP2 peptide having at least 9 amino acids and comprising the amino acid sequence $Xaa_1LPYWNFATXaa_2$ wherein Xaa_1 is any one of 20 naturally

occurring amino acids, preferably an amino acid selected from the group consisting of Ala, Gln, Val, Ser, Leu, Ile or no amino acid and Xaa_2 is any one of 20 naturally occurring amino acids, preferably Gly, or no amino acid, and analogs thereof which are recognized by immune cells in the context of HLA Class II to methods of preparing the specific T cells, and the use of the T cells as a diagnostic and therapeutic use.

Another aspect of the invention are antigen presenting cells (APC) expressing an appropriate MHC Class II molecule pulsed or transfected with the TRP2 peptide having at least 9 amino acids and comprising the amino acid sequence Xaa_1LPYWNFATXaa_2 wherein Xaa_1 any one of 20 naturally occurring amino acids, preferably is an amino acid selected from the group consisting of Ala, Gln, Val, Ser, Leu, Ile or no amino acid and Xaa_2 is any one of 20 naturally occurring amino acids, preferably Gly, or no amino acid, and analogs thereof which are recognized by immune cells in the context of HLA Class II or analogs thereof for use as a diagnostic or therapeutic reagent.

Another object of the invention is to provide a method of monitoring the efficacy of a cancer vaccine therapy in a mammal comprising (A) isolating T lymphocytes from the vaccine-treated mammal (B) measuring immunoreactivity of the T lymphocytes in the presence of the TRP2 peptide having at least 9 amino acids and comprising the amino acid sequence Xaa_1LPYWNFATXaa_2 wherein Xaa_1 is any one of 20 naturally occurring amino acids, preferably an amino acid selected from the group consisting of Ala, Gln, Val, Ser, Leu, Ile or no amino acid and Xaa_2 is any one of 20 naturally occurring amino acids, preferably Gly, or no amino acid, and analogs thereof, an enhancement of immunoreactivity in comparison to immunoreactivity of control lymphocytes is indicative of efficacy.

The present invention also relates to polyclonal, monoclonal and recombinant antibody elicited by and immunoreactive with, the TRP2 peptide having at least 9 amino acids and comprising the amino acid sequence Xaa_1LPYWNFATXaa_2 wherein Xaa_1 is any one of 20 naturally occurring amino acids, preferably an amino acid selected from the group consisting of Ala, Gln, Val, Ser, Leu, Ile or no amino acid and

Xaa_2 is any one of 20 naturally occurring amino acids, preferably Gly, or no amino acid, and analogs thereof for use as a diagnostic and/or therapeutic reagent.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A through 1F show the lysis of TRP2 (180-188) peptide pulsed target cells by bulk CTL from patients following 6 in vitro restimulations of the CTL with peptide-pulsed target cells. Specific lysis of control T2 cells(i.e. T2 cells not pulsed with peptide) was compared to that of T2 cells pulsed with 1 M TRP2 (180-188) by CTL from patients AN (a), MU (b), and KU (c) in 4 hr ^{51}Cr release cytotoxicity assays. In addition, specific lysis of 3 HLA-$A2^+TRP2^+$ melanoma cell lines was compared to that of 2 HLA-$A2^-TRP2^+$ and 1 HLA-$A2^+TRP2^-$ melanoma lines by bulk CTL from patients AN (d), MU (e), and KU (f).

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to the identification of HLA Class I or HLA Class II restricted peptides from the melanoma antigen, tyrosinase-related protein 2.

The invention relates to the identification of an HLA-A2 restricted peptide epitope, from the melanoma antigen tyrosinase-related protein 2 (TRP2). This peptide, designated TRP2 (180-188), was identified by screening TRP2 derived peptides for the ability to induce cytotoxic T lymphocytes (CTL) which specifically react with, and lyse, melanoma cells in the context of HLA-A*0201.

The invention therefore relates to the amino acid sequence of TRP2 (180-188), which is shown in SEQ ID NO: 1, or analogs thereof where the term analog as used throughout the specification and claims in context of HLA Class I molecules refers to sequences which are biologically equivalent to the native TRP2 (180-188) peptide in that they are able to induce cytotoxic T lymphocytes (CTL) which specifically react with, and lyse, melanoma cells in the context of HLA-A*0201.

Since human HLA-A2 molecules recognize peptides of nine or ten amino acids in length, TRP2 (180-189) (SVYDFFVWLH, shown as SEQ ID NO: 3) constitutes an analog of TRP2 (180-188). In addition, analogs of TRP2 (180-188)

include sequences in which one or more residues of SEQ ID NO:1 have been conservatively substituted such that the analog is biologically equivalent to the native TRP2 (180-188) peptide. Examples of conservative substitutions include the substitution of one-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for another, the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between glycine and serine, the substitution of one basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another. The phrase "conservative substitution" also includes the use of a chemically derivatized residue in place of a non-derivatized residue provided that the resulting peptide is biologically equivalent to the native TRP2 (180-188) peptide.

Preferred positions to be conservatively substituted in TRP2 (180-188) (SEQ ID NO:1) or its analog TRP2 (180-189) (SEQ ID NO: 3) include, but are not limited to, position 1 (residue 180), position 2 (residue 181) and the carboxy-terminal position (residue 188 in TRP2 (180-188) and residue 189 in TRP2 (180-189)). Preferred analogs of TRP2 (180-188) include, but are not limited to, substitution of either leucine or methionine at position 2 and the substitution of leucine for valine at the carboxy terminus.

The present invention further relates to the identification of an HLA Class II restricted peptide epitope from the melanoma antigen tyrosinase-related protein 2 and the equivalent peptide epitope from TRP-1. The HLA Class II restricted TRP2 peptides of the present invention are HLA-DR restricted, preferably HLA-DRB1*1501 or 1502 restricted. In one embodiment of the invention, the HLA Class II restricted TRP2 peptides of the present invention are expressed on melanomas derived from the majority of individuals that express the major histocompatibility complex (MHC) Class II allele HLA-DRB1*1501 or 1502, which is expressed in the Caucasian population at a frequency of between 9 an 19%. The HLA Class II restricted TRP2 peptides and analogs of the present invention are identified by the ability to induce helper T lymphocytes which specifically react with the peptide in the context of

peptide pulsed antigen presenting cells expressing the appropriate HLA-Class II molecule.

The invention provides a HLA Class II-restricted peptide of TRP2 comprising an amino acid sequence of at least about nine amino acids in length. The HLA Class II-restricted peptides of the present invention are as long as about 32 amino acids, preferably less than about 22 amino acids in length, more preferably between about 9 amino acids and about 16 amino acids in length. In one embodiment the peptide comprises the amino acid sequence Xaa_1LPYWNFATXaa_2 (SEQ. ID NO: 60) or analogs thereof, wherein Xaa_1 is any one of 20 naturally occurring amino acids, preferably an amino acid selected from the group consisting of Ala, Gln, Val, Ser, Leu, Ile or no amino acid; and Xaa_2 is any one of 20 naturally occurring amino acids, preferably Gly, or no amino acid.

The HLA Class II restricted TRP2 peptides of the present invention of the general formula, Xaa_1LPYWNFATXaa_2 may variably comprise one to about 11 additional amino acids at the N-terminus and/or one to about 11 additional amino acids at the C-terminus. In this embodiment, the HLA Class II restricted TRP2 peptide is represented by the formula, Xaa_3Xaa_1LPYWNFATXaa_2Xaa_4 (SEQ ID NO: 71) wherein Xaa_3 comprises from zero to about 11 amino acids in length, preferably from zero to about 16 amino acids; and Xaa_4 comprises variably from zero to about 11 amino acids in length, preferably from zero to about 16 amino acids in length. The amino acids for Xaa_3 and Xaa_4 may be any of the 20 naturally occurring amino acids.

In one embodiment Xaa_3 comprises the amino acid sequence Asp Leu Gln Arg Leu Ile Gly Asn Glu Ser Phe (SEQ ID NO: 72).

In one embodiment Xaa_4 comprises Arg. In yet another embodiment Xaa_4 comprises Arg Asn Glu Cys Asp Val Cys Thr Asp Gln Leu (SEQ ID NO: 73).

Other embodiments of the HLA Class II restricted TRP2 peptides invention comprise LPYWNFATG (SEQ. ID NO: 61); ALPYWNFAT (SEQ. ID NO: 62); ALPYWNFATG (SEQ. ID NO: 63); SLPYWNFATG (SEQ. ID NO: 64); QLPYWNFATG (SEQ. ID NO: 65); VLPYWNFATG (SEQ. ID NO: 66); ALPYWNFATGR (SEQ. ID NO: 67); FALPYWNFATG (SEQ. IF NO: 68);

LQRLIGNESFALPYWNFATG (SEQ. ID NO: 69); ALPYWNFATGRNECDVCTDQ (SEQ. ID NO: 70) and analogs of each sequence.

Analogs of the HLA-Class II restricted TRP2 peptides of the present invention are those peptides having one or more substitutions in the consensus binding motif which results in equivalent or enhanced immunological responses as compared to the native motif.

In one embodiment, an analog of the sequence ALPYWNFAT comprises a single substitution of Phe or Ile in place of Tyr or a single substitution of Ile, Leu, Val, or Met in place of Phe, or a combination of substitutions at each position.

In another embodiment, an analog of the sequence ALPYWNFAT comprises a single substiution of Phe, Tyr, or Ile in place of Trp or a single substitution of Ile, Leu, Val, Met, or Phe, in place of Ala, or combinations of substitutions at each position.

The HLA Class I restricted TRP2 peptides or analogs thereof and HLA Class II restricted TRP2 peptides or analogs thereof can be synthesized by automated instruments sold by a variety of manufactures or can be commercially custom-ordered and prepared. Alternatively, the peptide can be expressed from nucleic acid sequences which are capable of directing synthesis of the peptide using recombinant DNA methods known to those of ordinary skill in the art, and purified by methods known in the arts.

The present invention also encompasses a nucleic acid sequence encoding a HLA-Class I restricted peptide derived from TRP-2 or encoding an HLA-Class II restricted peptide derived from TRP-2. The nucleic acid sequence of the entire native TRP2 gene is disclosed in U.S. Patent No. 5,831,016 incorporated herein by reference.

The invention therefore relates to the nucleic acid sequence encoding TRP2 (180-188) and its analogs with a preferred nucleic acid sequence shown as SEQ ID NO: 2 (AGTGTTTATGATTTTTTTGTGTGGCTC).

The invention further relates to the nucleic acid sequence encoding an

HLA-Class II restricted TRP2 peptide. In one embodiment the nucleic acid sequence encodes a peptide comprising Xaa_1LPYWNFATXaa_2 or analogs thereof, wherein Xaa_1 is any one of 20 naturally occurring amino acids, preferably an amino acid selected from the group consisting of Ala, Gln, Val, Ser, Leu, Ile or no amino acd; and Xaa_2 is any one of 20 naturally occurring amino acids, preferably Gly, or no amino acid.

It should be noted that the nucleic acid described herein represent a preferred embodiment of the invention. Due to the degeneracy of the genetic code, it is to be understood that numerous choices of nucleotides may be made that will lead to a sequence capable of directing production of the HLA Class I restricted TRP2 (180-188) peptide or analogs thereof and the HLA Class II restricted TPR2 peptide or analog thereof. As such, nucleic acid sequences which are functionally equivalent to the sequences described herein are intended to be encompassed within the present invention.

The invention further relates to expression vectors comprising the nucleic acid sequences encoding the HLA Class I restricted TRP2 (180-188) or analogs thereof or comprising the nucleic acid sequences encoding the HLA-Class II restricted TRP2 peptide or analogs thereof. Any expression vector that is capable of carrying and expressing the nucleic acid sequences encoding the HLA Class I restricted TRP2 (180-188) or the HLA Class II restricted TRP2 peptides or analogs thereof in prokaryotic or eukaryotic host cells may be used including but not limited to recombinant virus such as vaccinia, fowlpox or adenovirus and the like. The invention also encompasses host cells transformed, transfected or infected with the vector to express the HLA-Class II restricted TRP2 peptide or analog thereof. The host cell may endogenously express an appropriate HLA-Class II molecule or may be recombinantly engineered to express an exogenous HLA Class II molecule, using methods known in the art.

As HLA-A2 is the most commonly expressed family of class I MHC molecules in melanoma patients in the United States with an estimated frequency of 47% and HLA-A*0201 is the most common subtype, being expressed in approximately 98% of the HLA-A2 positive patients in North America. The MHC Class II allele HLA-DRB1*1501 and 1502 is expressed in the Caucasian population at a frequency of between 9 and 19%. As such, the HLA Class I and HLA Class II restricted TRP2

peptides may be used to screen for melanomas. Therefore, the present invention also relates to the use of the HLA-Class I restricted TRP2 (180-188) peptide and/or analogs thereof and use of HLA-Class II restricted TRP2 peptides in a method for detecting the presence of melanoma in a mammal, where, as used in this application, the term melanoma includes, but is not limited to, melanomas, metastatic melanomas, melanomas derived from either melanocytes, melanocarcinomas, melanoepitheliomas, melanosarcomas, melanoma in situ, superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma, acral lentiginous melanoma, invasive melanoma or familial atypical mole and melanoma (FAM-M) syndrome.

In one embodiment, the method of detecting melanoma involves isolating cytolytic T lymphocytes (CTLs) from peripheral blood, lymph nodes or spleens of a mammal using methods known to those skilled in the art and, coincubating the CTLs in vitro with target cells pre-exposed to either TRP2 (180-188) peptide and/or analogs thereof, or to expression vector containing the nucleic acid sequences encoding the TRP2 (180-188) peptide or analogs thereof. Ratios of CTL to target cells to be used in the method range from about 1:1 to about 100:1.

Target cells to be used in the method include, but are not limited to, HLA-$A2^{+}TRP2^{+}$ melanoma cell lines, HLA-$A^{*}0201^{+}$ T2 cells pulsed with TRP2 (180-188) and/or analogs thereof, or antigen presenting cells such as B cells, dendritic cells, macrophages, Langerhan cells pulsed with TRP2 (180-188) and/or analogs thereof as defined above. Target cells also include but are not limited to HLA-DRB1*1501^{+} or 1502^{+} B cells, melanoma cells, antigen presenting cells and the like pulsed with a HLA Class II restricted TRP2 peptide or analog thereof or recombinantly expressing the HLA-Class II restricted TRP peptide or analog thereof.

The helper T cell response or CTL response to the target cells can be determined by a variety of methods including measuring cytokine release by the helper T cells or CTLs following coincubation with the target cells or by measuring lysis of the target cells by the CTLs.

Where cytokine release by the helper T cells or CTLs is to be utilized as an indicator of the production of a specific helper T cell or specific CTL response to the

TRP2 peptides or analogs thereof, the cytokine release can be measured by methods known to those skill in the art including immunoassays such as ELISAs and radioimmunoassays.

A preferred cytokine to assay for release from stimulated CTLs is IFN as described in the Examples. Other cytokines which can be measured include, but are not limited to, GM-CSF, TNF and IL-2.

Where the lytic activity of the CTLs is to be measured as an indicator of a TRP2 (180-188) peptide specific CTL response following coincubation with target cells, the lysis of target cells can be measured using methods known to those skilled in the art including the ^{51}Cr-release assay described in Example 2.

To determine whether the patient has melanoma, cytokine release by the CTLs or specific lysis of target cells by CTLs as measured above is then compared to the cytokine secretion or lysis of target cells by CTLs coincubated in vitro with target control cells where control cells include, but are not limited to, HLA-A2$^-$TRP2$^+$ or HLA-A2$^+$TRP2$^-$ cells, or T2 cells or dendritic cells not exposed to peptide. A two-fold or more increase in cytokine release or cytolytic activity as compared to that of the control cells indicates the presence of melanoma.

The present invention also relates to methods of treating a mammal having melanoma. In one embodiment, the method comprises exposing T lymphocytes, preferably autologous cytotoxic lymphocytes or tumor infiltrating lymphocytes obtained from a patient with melanoma, in vitro to a TRP2 peptide and/or analogs thereof, either alone or in combination with other HLA-A2-restricted epitopes from TRP 2, or in combination with other HLA-DR restricted epitopes from TRP2, or other melanoma antigens to elicit TRP2 specific CTLs or helper T lymphocytes, and administering the TRP2 specific CTLs or helper T lymphcytes to the mammal.

It is believed that since HLA-A*0201 restricted epitopes have been identified to date from 6 known melanoma antigens: MART-1 (17), gp100 (6, 7, 18-20), tyrosinase (21), MAGE-3 (22, 23), N-acetylglucosaminyl-transferase-V (GnT-V) (24), and the melanocyte-stimulating hormone receptor MC1R (4) (the epitopes disclosed in references 4, 6, 7 and 17-24 are hereby incorporated by reference),

immunotherapies utilizing HLA-A*0201 specific antigens are applicable to a large number of patients.

Techniques for sensitizing human T lymphocytes in vitro to tumor antigen immunodominant peptides are known in the art. In addition, lymphocytes can be subjected to repetitive in vitro stimulation to produce peptide-specific lymphocytes having a greater capacity to recognize human tumor antigens.

In another embodiment, the method of treating a mammal having melanoma comprises immunizing the mammal with the HLA Class I restricted TRP2 peptide and/or analogs thereof in an amount effective to elicit a HLA Class I restricted TRP2 (180-188) peptide-specific response. In one embodiment, the TRP2 (180-188) peptide and/or analogs thereof can be fused with an endoplasmic reticulum signal peptide as described in U. S. patent 5,733,548, hereby incorporated by reference, and the resulting chimeric protein administered to a mammal in an amount effective to prevent melanoma.

In an embodiment, the method of treating a mammal having a melanoma comprises immunizing the mammal with a HLA-Class II restricted TRP2 peptide, analog, or combination thereof in an amount effective to elicit a HLA-Class II restricted TRP2 peptide specific response. The method may further provide cytokines such as IL-2, IL-4, and the like, or adjuvants such as RIBI-Detox™, alum, and the like for enhancement of the immune response. The HLA-Class II restricted TRP2 peptides of the present invention may also be provided in the form of a TRP2 peptide-protein carrier conjugate, for enhancing the immune response. Protein carriers which may be used include but are not limited to Pseudomonas exotoxin, poly-L-lysine and the like as are known in the art.

In an alternative embodiment, an expression vector containing the nucleic acid sequences encoding the TRP2 peptide or analogs thereof may be administered to the mammal having melanoma in an amount effective to elicit a TRP2 peptide-specific response. Of course, one of skill in the art would recognize that the TRP2 peptide or nucleic acid sequences encoding the peptide could be administered to the mammal in combination with other HLA-A2 restricted epitopes or other HLA-Class II restricted

epitopes from TRP2 or melanoma antigen such as those identified in MART-1, gp100, tyrosinase, MAGE-3, GnT-V and MC1R as disclosed above.

CTL populations or helper T lymphocyte populations reactive against the TRP2 peptide may then be isolated from a peripheral blood sample or spleen cells of the mammal immunized with the peptide or expression construct from about 3 to about 30 days after immunization. Epstein-Barr virus (EBV) can be used to immortalize human lymphocytes or a human fusion partner can be used to produce human-human hybridomas.

CTLs or helper T lymphocytes are cultured for about 7 to about 90 days (3) and then screened to determine the clones of the desired reactivity against the TRP2 peptide using known methods of assaying T cell reactivity; CTLs or helper T lymphocytes producing the desired reactivity are thus selected.

The selected CTLs or helper T lymphocytes may be administered via one of several routes including but not limited to intravenous, intraperitoneal, intramuscular or subcutaneous. A preferred route of administration is intravenously.

In general, it is desirable to provide the recipient with a dosage of about 10^7 to about 10^{11} CTLs or helper T lymphocytes. A preferred dosage is about $5x10^9$ to about $5x10^{10}$ lymphocytes.

The invention further relates to CTLs or helper T lymphocytes having specific reactivity to the TRP2 peptide, and to the use of such CTLs or helper T lymphocytes as diagnostic and therapeutic agents. For example, TRP2 (180-188) specific CTLs can be used diagnostically to screen target cells (i.e. antigen presenting cells such as dendritic cells, macrophages and Langerhan cells) from a patient by measuring lysis of target cells following coincubation with the peptide-specific CTLs, wherein lysis of the target cells indicates that the patient has melanoma.

Alternatively, TRP2 specific CTLs or helper T lymphocytes can be used prognostically to detect HLA-$A2^+TRP2^+$ target cells or HLA-DRB1*1501^+ or 1502^+ $TRP2^+$ target cells. If such target cell were isolated from a patient's tumor and were recognized by the TRP2 specific CTLs or helper T lymphocytes, the indication would be that the tumor was a melanoma.

In yet another embodiment, the method of treating a mammal having melanoma comprises administering target cells which have been exposed in vitro to the TRP2 (180-188) peptide and/or analogs thereof or the MHC Class II restricted TRP2 peptide or analogs thereof to a mammal in an amount effective to elicit a specific T lymphocyte response to the TRP2 peptides. For example, dendritic cells cultured for about 1 week in vitro with about 1000 u/ml GM-CSF and 1000 u/ml IL-4 and pulsed with the TRP2 peptide and/or analogs thereof can be administered intravenously to a mammal at a dosage of about $1x10^8$ to about $2x10^8$ cells.

The invention therefore also relates to target cells which have been exposed in vitro to the TRP2 (180-188) peptide and/or analogs thereof or the MHC Class II restricted TRP2 peptide or analogs thereof. These target cells can be used as diagnostic and therapeutic reagents. For example, target cells which have been incubated in vitro with the TRP2 (180-188) peptide and/or analogs thereof can be used diagnostically to screen CTLs from a patient by measuring lysis of the target cells following incubation with the CTLs, wherein lysis of the target cells indicates that the patient has melanoma. Or in the case of an MHC Class II restricted TRP2 peptide or analog thereof, target cells which have been incubated in vitro with the peptide may be used to screen for peptide-specific helper T lymphocytes from a patient by measuring IFN-γ release.

The present invention further relates to a method of preventing melanoma in a mammal, said method comprising administering to the mammal an effective amount of the TRP2 (180-188) peptide and/or analogs thereof, alone or in combination with other HLA-A2-restricted epitopes from TRP2 or other melanoma antigens in an amount effective to prevent melanoma in the mammal.

The present invention also relates to a method of preventing melanoma in a mammal, comprising administration of an effective amount of an HLA Class II restricted TRP2 peptide and/or analog thereof.

The present invention further relates to a method of preventing melanoma in a mammal, said method comprising administering to the mammal an effective amount of an expression vector containing nucleic acid sequences encoding the TRP2 (180-188)

peptide and/or analogs thereof, alone or in combination with other HLA-A2-restricted epitopes from TRP2 or other melanoma antigens in an amount effective to prevent melanoma in the mammal.

The present invention also relates to a method of preventing melanoma in a mammal, said method comprising the administration of an effective amount of an expression vector containing nucleic acid sequences encoding an HLA-Class II restricted TRP2 peptide or analog thereof to prevent melanoma in the mammal.

In another embodiment, the nucleic acid sequence encoding at least one MHC Class II restricted peptide may be used directly as an immunogen or vaccine using techniques utilizing "naked" DNA which is directly injected into muscle or skin, or linked to a lipid molecule.

After immunization the efficacy of the vaccine can be assessed by production of immune cells that recognize the tumor antigen, as assessed by specific lytic activity, specific cytokine production, tumor regression or a combination of these approaches. If the mammal to be immunized is already afflicted with melanoma or metastatic melanoma, the vaccine can be administered in conjunction with other molecules such as immunomodulators, for example, IL-2, IL-6, IL-10, IL-12, IL-15, interferon, tumor necrosis factor and the like, adjuvants, chemotherapeutic drugs such as cisplatinum, antiviral such as gancyclovir, amphotericin B, antibiotics and the like.

In the methods of preventing or treating melanoma, the HLA Class I restricted TRP2 (180-188) peptide and/or analogs thereof or the HLA-Class II restricted TRP2 peptide and/or analog thereof may be administered via one of several routes including but not limited to subcutaneous, intradermal, intramuscular, intrathecal, intrapleural, intrauterine, rectal, vaginal, topical, intratumor and the like.

Administration may also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be by nasal sprays, for example, or suppositories.

For oral administration, the TRP2 peptide or analog thereof is formulated into conventional oral administration form such as capsules, tablets. A preferred route of administration of peptide is via subcutaneous injection in IFA (incomplete Freund's adjuvant).

In general, it is desirable to provide the recipient with a dosage of TRP2 peptide or analogs thereof effective to prime, stimulate and/or cause the clonal expansion of peptide-specific T lymphocytes and/or helper T lymphocytes, preferably cytotoxic T lymphocytes, which in turn are capable of preventing or inhibiting melanoma in the recipient. A preferred dosage of TRP2 peptide or an analog thereof is of at least about 1 pg per kg bodyweight, more preferably at least about 1ng per kg bodyweight, and most preferably at least about 1μg or greater per kg bodyweight of the recipient.

When nucleic acid of the invention is utilized in the method of preventing or treating melanoma, preferred routes of administration are intramuscularly and intradermally, and vectors containing the sequences are administered at a dosage of about 1 to about 10 mg.

The dose of peptide or nucleic acid is administered at least once and may be provided as a bolus or a continuous administration. Multiple administrations of the dose over a period of several weeks to months may be preferable. Subsequent doses may be administered as indicated. In general, it is desirable to provide DNA vectors at a dosage of about 1 to about 10 mg, at about 4 week intervals, and peptides at a dosage of about 1 mg, at about 3-4 week intervals.

The present invention also relates to a pharmaceutical composition comprising an HLA-Class I or HLA-Class II restricted TRP2 peptide and/or analogs thereof, alone or in combination with different HLA-A2-restricted epitopes from TRP2, or one or more different HLA-Class II restricted epitopes from TPR2, a combination thereof, or other melanoma antigens.

The present invention also relates to a pharmaceutical composition comprising an expressing vector comprising the nucleic acid sequence encoding an HLA-Class I or HLA-Class II restricted TRP2 peptide and/or analogs thereof, alone or in

combination with HLA-A2-restricted epitopes from TRP2, an HLA-Class II restricted epitope from TRP2, or other melanoma antigens.

The TRP2 peptides of this invention or analogs thereof may be formulated alone or with any other HLA-A2-restricted epitopes from TRP2, an HLA-Class II restricted epitope from TRP2, or other melanoma antigens with pharmaceutically acceptable carriers into pharmaceutical compositions by methods known in the art. The compositions may further comprise at least one immunostimulatory molecule where immunostimulatory molecules to be used in conjunction with the TRP2 peptide for stimulating antigen specific T cell responses include, but are not limited to, one or more major histocompatibility complex (MHC) molecules, such as class I and class II molecules. The composition may further comprise other stimulator molecules including B7.1, B7.2, ICAM-1, ICAM-2, LFA-1, LFA-3, CD72 and the like, and cytokines which include but are not limited to IL-1 through IL-15, TNFα, IFNγ, RANTES, G-CSF, M-CSF, IFNα, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1β, or combination thereof, and the like for immunopotentiation.

The present invention also encompasses antibody elicited by, and immunoreactive with, the HLA-Class II restricted TRP2 peptides. The antibody may be polyclonal, monoclonal, chimeric, or recombinant antibody. Recombinant antibody includes, but is not limited to single chain antibody which may be made by methods known in the art. The antibody of the present invention has utility as a diagnostic reagent in assays to detect cancer cells and in assays to monitor cancer vaccine therapy. The antibody may be provided in kit form along with other standard reagents for immunoassays. The antibody of the present invention may be used therapeutically in the form of a pharmaceutical composition to inhibit the growth of cancer cells expressing TRP2 peptides.

The present invention will now be described by way of examples, which are meant to illustrate, but not limit, the scope of the invention.

All of the patents and references cited herein are hereby incorporated by reference.

Examples

Materials and Methods

Cell culture

The Skmel23 human melanoma cell line was kindly provided by Thierry Boon (Ludwig Institute for Cancer Research, Brussels, Belgium), and A375 was purchased from American Type Culture Collection (Rockville, MD). All other human melanoma cell lines were established in our laboratory (31). Melanoma cell lines and T2 cells (HLA-A*0201$^+$ peptide transporter associated protein deficient T cell-B cell hybrid-(32) were routinely cultured in RPMI1640 (Mediatech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biofluids, Rockville, MD) and 2 mM L-glutamine (Biofluids). The COS-7 monkey kidney cell line was kindly provided by W. Leonard (National Institutes of Health) and was maintained in DMEM (Mediatech or Biofluids) containing 10% heat-inactivated FBS, 2 mM L-glutamine, and 10 mM HEPES. Human lymphocytes were cultured in complete medium (CM) consisting of Iscove's modified DMEM with 25 mM HEPES, 2 mM L-glutamine, 50 U/ml penicillin, 50 mcg/ml streptomycin (Biofluids), and 10% heat-inactivated human AB serum (Sigma Chemical Co., St. Louis, MO; Valley Biomedical, Winchester, VA).

The presence of TRP2 mRNA in melanoma cell lines was previously assessed by Northern Blot analysis (13). The expression of HLA-A2 was evaluated by FACS using an anti-HLA-A2 monoclonal antibody (One Lambda, Canoga Park, CA), and for some cell lines, DNA sequencing confirmed the presence of HLA-A*0201 (HLA laboratory, National Institutes of Health, Bethesda, MD). The expression of TRP2, HLA-A2 or HLA-A*0201 in melanoma cell lines was as follows: 397mel (HLA-A2$^-$, TRP2$^+$), 888mel (HLA-A2$^-$, TRP2$^+$), A375 (HLA-A*0201$^+$, TRP2$^-$), 526mel (HLA-A2$^+$, TRP2$^+$), 624mel (HLA-A*0201$^+$, TRP2$^+$), 1300mel (HLA-A2$^+$, TRP2$^+$), Skmel23 (HLA-A*0201$^+$, TRP2$^+$), and 501mel (HLA-A*0201$^+$, TRP2$^+$). In addition, COS-7 cells expressing HLA-A*0201 and TRP2 or MART-1 were generated by co-transfection (Lipofectamine Plus; Gibco) with cDNAs encoding these proteins (pCDNA3 plasmid; Invitrogen).

*Peptide synthesis and HLA-A*0201 binding affinity assays*

Candidate peptides were selected from TRP2 which conformed to a permissive HLA-A*0201 binding motif were selected from TRP2 based on the following set of criteria: (1) peptides which bind with high affinity to HLA-A*0201 are generally 9 or 10 amino acids in length and contain L or M at the second position from the amino-terminus (P2) and V or L at the carboxy-terminus (P9 or P10) (30); (2) amino acids at secondary anchor positions, most notably P1 and P3, can significantly affect peptide binding to HLA-A*0201 (28; 29); and (3) many previously identified HLA-A*0201 restricted epitopes from nonmutated melanoma antigens are 9 amino acids in length and have a dominant residue at P2 or the C-terminus, but not both primary anchor positions (37; 38). Based on these observations, 9-mers were chosen which contained L/M at P2 or V/L at P9; 10-mers were selected with L/M at P2 and L/V at P10; and peptides with D, E, P, R, or H at P1 were eliminated. In total, 44 9-mers and 7 10-mers were synthesized (Table 1).

TRP2 peptides and the FluM1 peptide (influenza matrix protein (58-66): GILGFVFTL: SEQ ID NO: 57) were initially synthesized using a solid-phase method based on fluorenylmethoxycarbonyl (Fmoc) chemistry on a multiple peptide synthesizer (model AMS 422; Gilson Co., Worthington, OH), and molecular weights were verified by laser desorption mass spectrometry (Bio-Synthesis, Inc., Lewisville, TX). MART-1(27-35) (AAGIGILTV: SEQ ID NO: 58) was commercially synthesized, purified (>95%), and characterized by amino acid analysis (Peptide Technologies, Gaithersburg, MD).

The relative binding affinity of each TRP2 peptide to HLA-A*0201 was experimentally determined on the basis of the inhibition of binding of a standard radiolabeled peptide to purified MHC molecules as previously described (33). Briefly, the test peptide was coincubated at various concentrations (1 nM to 100 mM) with soluble HLA-A*0201 heavy chain, human β_2-microglobulin, and 5 nM ^{125}I-labeled HBc (18-27) with Y substituted at P6 (FLPSDYFPSV: SEQ ID NO: 59). The concentration of the test peptide necessary to inhibit the binding of the iodinated peptide by 50% (ID_{50}) was calculated.

In later experiments, TRP2 (217-225) was purified (>95%) by reverse-phase HPLC on a POROS 10 column (PerSeptive Biosystems, Cambridge, MA) using a 0.05% trifluoroacetic acid/water-acetonitrile gradient, and the molecular weight was verified by mass spectrometry (Bio-Synthesis, Inc.). TRP2(180-188) and the FluM1 peptide were commercially synthesized, purified (>95%), and characterized by amino acid analysis (Peptide Technologies).

The binding affinities of TRP2 peptides to HLA-A*0201 were evaluated on the basis of the inhibition of binding of a standard radiolabeled peptide to purified MHC molecules (33). The concentration of test peptide necessary to inhibit the binding of the standard peptide by 50% (ID_{50}) was calculated, and peptide binding affinity was defined as high (ID_{50}<50 nM), intermediate (50 nM<ID_{50}<500 nM), or weak (500 nM<ID_{50}).

Cytokine release and cytotoxicity assays

Recognition of TRP2 by bulk T cell cultures was evaluated about 7 days after each stimulation on the basis of IFNγ secretion by CTLs in response to T2 cells preincubated with peptide, HLA-$A2^+$ $TRP2^+$ melanoma cells, or, in some experiments, COS-7 cells expressing HLA-A*0201 and TRP2. T2 cells were incubated with peptide 1 to 3 hr at 37° C and were either used directly (peptide-loaded) or were washed twice prior to use (peptide-pulsed). 10^5 responder T cells were coincubated with 10^5 stimulator cells (250 µl total) about 20 hr at 37° C, and the concentration of human IFNγ in coculture supernatants was measured using a commercially available ELISA kit (Endogen, Cambridge, MA).

In some experiments, 4 hr ^{51}Cr release cytotoxicity assays were also performed to evaluate the recognition of TRP2 by bulk CTL as previously described (36). Briefly, ^{51}Cr-labeled T2 cells were incubated with 1 µM peptide for about 1 hr at 37° C and washed twice. These cells and ^{51}Cr-labeled melanoma cells were coincubated with effector cells (peptide stimulated CTLs) (5000 targets/well; multiple E:T ratios;150 µl total) 4 hr at 37° C, and the radioactivity in coculture supernatants was determined by gamma counting. The percent specific lysis of target cells by CTL

was calculated, and spontaneous ^{51}Cr release from target cells never exceeded 20% of the maximum.

Table 1
Binding Affinities of TRP2 Peptides to HLA-A*0201

Peptide	Sequence ID No:	Sequence	ID_{50} (nM)[1]
431-439	5	NMVPFFPPV	4
185-193	6	FVWLHYYSV	15
455-463	7	YAIDLPVSV	16
288-296	8	SLDDYNHLV	26
482-490	9	ALVGLFVLL	26
476-484	10	VMGTLVALV	28
479-488	11	TLVALVGLFV	29
180-188	12	SVYDFFVWL	36
475-483	13	VVMGTLVAL	67
156-164	14	YVITTQHWL	104
217-225	15	VTWHRYHLL	161
36O-368	16	TLDSQVMSL	161
367-376	17	SLHNLVHSFL	263
472-481	18	TLLVVMGTLV	357
234-242	19	LIGNESFAL	385
394-402	20	VLHSFTDAI	417
364-372	21	QVMSLHNLV	676
216-224	22	FVTWHRYHL	943
473-481	23	LLVVMGTLV	1351
241-249	24	ALPYWNFAT	1351
489-498	25	LLAFLQYRRL	1515
472-480	26	TLLVVMGTL	2632
450459	27	QLGYSYAIDL	2632
9-17	28	LLSCLGCKI	3571
385-393	29	SAANDPIFV	4545
478-486	30	GTLVALVGL	4545
28-36	31	VCMTVDSLV	7143
481-489	32	VALVGLFVL	7143
406-414	33	WMKRFNPPA	8333
20-28	34	GAQGQFPRV	>10,000
56-64	35	QGRGQCTEV	>10,000
117-125	36	NCERKKPPV	>10,000
125-133	37	VIRQNIHSL	>10,000
144-152	38	ALDLAKKRV	>10,000
158-166	39	ITTQHWLGL	>10,000
159-167	40	TTQHWLGLL	>10,000

[1] Peptide binding affnity to HLA-A*0201 was evaluated by measuring the concentration of peptide necessary to inhibit the binding of a standard radiolabeled peptide by 50% (IDso). Relative binding affnity was defined as high (I DsO<5O nM), intermediate (5O n M< I DsO<5OO nM), or weak (ID50>500 nM).

Peptide	Sequence ID No:	Sequence	ID_{50} (nM)[1]
163-171	41	WLGLLGPNG	>10,000
178-186	42	NCSVYDFFV	>10,000
226-234	43	CLERDLQRL	>10,000
248-256	44	ATGRNECDV	>10,000
264-272	45	AARPDDPTL	>10,000
311-319	46	QMGRNSMKL	>10,000
321-329	47	TLKDIRDCL	>10,000
343-351	48	STFSFRNAL	>10,000
386-394	49	AANDPIFVV	>10,000
480-488	50	LVALVGLFV	>10,000
485-493	51	GLFVLLAFL	>10,000
490-498	52	LAFLQYRRL	>10,000
502-510	53	YTPLMETHL	>10,000
9-18	54	LLSCLGCKIL	>10,000
76-87	55	ILRNQDDREL	>10,000

Example 1

Identification of HLA-A*0201 Binding Peptides From TRP2

Of the 51 TRP2 peptides which fit the extended HLA-A*0201 binding motif as described in the Methods section, only 16 actually bound to HLA-A*0201 with high (ID_{50}<50 nM)or intermediate affinity (50 nM<ID_{50}<500 nM) (Table 1).

Example 2

CTL Induction with TRP2 Synthetic Peptides In Vitro

The 21 TRP2 peptides with ID_{50}<2000 nM (Table 1) were used to stimulate peripheral blood lymphocytes (PBL) in vitro from 4 HLA-A*0201$^+$ patients with metastatic melanoma.

In an initial screening of peptides for the induction of tumor reactive CTL, the 21 TRP2 peptides and MART-1(27-35) (as a positive control) were used to stimulate lymphocytes in vitro from 4 HLA-A*0201$^+$ melanoma patients as follows: T cell cultures were established by plating peripheral blood mononuclear cells (PBMC) in 24 well plates ($1.5x10^6$ cells/ml; 2 ml/well) in CM containing peptide (5 μg/ml for TRP2 peptides or 2 μg/ml for MART-1(27-35)) and GM-CSF (200 U/ml; PeproTech, Rocky Hill, NJ) and IL-4 (100 U/ml; PeproTech) to promote the differentiation of dendritic cells (34). Seven days later, 300 IU/ml of rIL-2 (Chiron Co., Emeryville, CA) was added. On day 11 and weekly thereafter, lymphocytes were restimulated with peptide-pulsed autologous PBMC as previously described (35).[2] IFNγ secretion in response to peptide-loaded T2 cells and HLA-A2$^+$ TRP2$^+$ melanomas was measured 7 days after the third and fourth restimulations (days 32 and 39).

Lymphocytes from 3 of 4 patients proliferated during this study, and for a given patient, no significant or consistent differences were observed between lymphocyte expansions with different peptides. Peptide recognition by bulk T cell

[2] Briefly, responder lymphocytes were harvested and replated in new 24-well plates ($2.5x10^5$ cells/ml; 2 ml/well) in CM. Autologous irradiated (3000 rad) PBMC were incubated with peptide (5 μg/ml for TRP2 peptides or 2 μg/ml for MART-1(27-35)) in 15 ml conical tubes ($1-3x10^6$ cells/ml; 6-12 ml/tube) 2 to 4 hours at 37° C. Peptide-loaded PBMC were washed and added to responder lymphocytes at a responder to stimulator ratio of about 1:7. One day after each restimulation, 150 IU/ml rIL-2 was added; and generally, cultures were split 1:1 two to three days later with CM containing 300 IU/ml rIL-2.

cultures after 4 restimulations is presented in Table 2. In patients HU and IN, TRP2 (431-439), TRP2 (180-188), TRP2 (217-225), and MART-1 (27-35) induced peptide reactive T cells, and in patient CA, peptide specific CTL were generated with TRP2 (476-484) and TRP2 (217-225). However, no TRP2 peptide induced CTL specifically recognized HLA-$A2^+$ $TRP2^+$ melanoma cells; only T cells from patients HU and IN stimulated with the positive control peptide MART-1 (27-35) specifically secreted IFNγ in response to HLA-$A2^+$ melanomas (data not shown).

One possible explanation for the lack of melanoma recognition by bulk T cells stimulated with TRP2 peptides was that these cultures contained only small numbers of high avidity T cells capable of recognizing low levels of TRP2 peptides expressed on the surfaces of melanoma cells. If this were the case, melanoma reactivity might be apparent in T cell clones derived from peptide specific cultures.

However, since these T cells were unavailable for further analysis, PBL from additional HLA-$A*0201^+$ melanoma patients were stimulated in vitro with the two TRP2 peptides which most efficiently induced peptide-reactive CTL in the initial screening: PBL from 4 HLA-$A*0201^+$ melanoma patients (MU, KU, AN, and WE) were stimulated in vitro with TRP2(180-188), and PBL from 4 separate patients (MC, WO, LE, and CA) were stimulated with TRP2(217-225) using a standard protocol which had previously been used successfully to generate melanoma reactive CTL with peptides from MART-1 and gp100 (40, 41).

In particular, PBMC were initially cultured in culture medium containing 1 μM peptide (instead of 5 μg/ml) without GM-CSF and IL-4, and 300 IU/ml IL-2 was added two days later. The lymphocytes were then restimulated weekly with peptide-pulsed autologous PBMC as described above (see footnote 2) beginning at day 7 (instead of day 11as in the initial screening), except that the PBMC were added to the responsive lymphocytes at a responder to stimulator ratio of about 1:10. IFNγ secretion in response to peptide-loaded T2 cells, COS-7 cells expressing HLA-A*0201 and TRP2, and HLA-$A2^+$ $TRP2^+$ melanoma cells was measured about 7 days after each stimulation beginning at one week.

Table 2 Initial Screening of TRP2 Peptides for Induction of Peptide Reactive CTL*

	Patient HU			Patient IN			Patient CA		
Peptide used for sensitization	media‡	FluM1§	relevant Peptide¶	media	FluM1	Relevant peptide	media	FluM1	relevant peptide
TRP2(431-439)	21	22	181	12	8	84	0	7	4
TRP2(185-193)	16	24	27	21	11	16	128	151	196
TRP2(455-463)	32	29	24	9	8	21	44	23	34
TRP2(288-296)	7	9	22	16	11	10	2	4	6
TRP2(482-490)	4	6	5	10	20	20	7	46	52
TRP2(476-484)	1	2	3	3	3	3	1	2	1048
TRP2(479-488)	15	16	14	11	11	13	2	3	3
TRP2(180-188)	18	18	337	16	6	898	2	0	0
TRP2(475-483)	7	8	11	7	3	3	4	5	4
TRP2(156-164)	33	22	33	42	15	13	1	1	0
TRP2(217-225)	5	7	>2000	8	10	>2000	1	5	>2000
TRP2(360-368)	6	8	8	9	28	34	0	3	3
TRP2(367-376)	8	7	7	6	7	9	6	5	3
TRP2(472-481)	12	6	7	8	7	8	2	3	2
TRP2(234-242)	71	54	54	3	3	5	3	0	1
TRP2(394-402)	2	2	4	8	11	7	1	2	1
TRP2(364-372)	7	9	10	15	17	16	4	4	2
TRP2(216-224)	65	60	52	8	6	6	0	3	2
TRP2(473-481)	6	4	4	16	14	16	2	2	2
TRP2(241-249)	11	11	13	24	19	19	290	236	251
TRP2(489-498)	22	23	23	5	8	6	4	6	7
MART-1(27-35)	9	7	1762	3	4	>2000	5	4	23

*IFNγ release (pg/ml) in 20 hr coculture supernatants of stimulators with bulk T cells after 4 in vitro restimulations with the indicated peptide.

‡media: IFNγ release in the absence of stimulators.

§FluM1: IFNγ release in response to T2 cells preincubated with 5 g/ml FluM1 peptide.

¶relevant peptide: IFNγ release in response to T2 cells preincubated with 5 g/ml of the peptide used for PBL sensitization.

**underlined values indicate that IFNγ release in response to T2 cells preincubated with the relevant peptide was ≥50 pg/ml and at least twice background with either media or T2 cells pre-loaded with the FluM1 peptide.

Specific peptide recognition by bulk CTL stimulated with TRP2(217-225) was apparent as early as week 3 from one patient (WO), and by week 5, T cell cultures from 3 of the 4 patients (MC, WO, and LE) specifically released IFNγ in response to peptide-loaded T2 cells. Similar to the initial TRP2 peptide screening, none of the bulk cultures stimulated with TRP2(217-225) recognized COS-7 transfectants or HLA-A2$^+$ TRP2$^+$ melanoma cells (data not shown).

TRP2 (180-188) induced peptide reactive T cells from 2 of 4 patients (KU and MU) after only 2 restimulations (week 3). More significantly, at week 3, bulk CTL from patient KU specifically secreted IFNγ when cocultured with COS-7 cells expressing HLA-A*0201 and TRP2 and HLA-A2$^+$ TRP2$^+$ melanoma cells. By week 5, the same pattern of recognition was observed by CTL from patient MU. Based on these observations, lymphocytes from the 3 patients whose cells proliferated (AN, KU, and MU) were restimulated, and by week 7, bulk CTL from all three patients specifically recognized peptide-pulsed T2 cells, COS-7 cells expressing HLA-A*0201 and TRP2, and HLA-A2$^+$ TRP2$^+$ melanomas (Table 3).

To determine if recognition of melanoma cells correlated with that of low concentrations of peptide, IFNγ release by CTL was measured in response to T2 cells pulsed with various concentrations of TRP2 (180-188) between 10^{-5} and 10^{-12} M. Peptide-induced CTL from patients KU and MU recognized TRP2 (180-188) pulsed on T2 cells at a concentration of 10^{-9} M, whereas T cells from patient AN did not specifically release IFNγ at a peptide concentration below 10^{-7} M. This correlated with specific tumor recognition in that IFNγ release by T cells from patient AN in response to HLA-A2$^+$ TRP2$^+$ melanoma cells was generally lower than that from patients KU and MU (Table 3).

Table 3

Recognition of Peptide, CoS-7 Transfectants. and Melanoma Cells by PBL from HLA-A*0201$^+$ Melanoma Patients Stimulated with TRP2(180-188)*

	Patient: MU	Patient: KU	Patient: AN
Media	0	12	0
T2 + nopeptide	0	8	0
T2 + 1 μM FluM1	0	nt‡	0
T2 + 10^{-12} M TRP2(180-188)	0	nt	0
T2 + 10^{-11} M TRP2(180-188)	2	nt	0
T2 + 10^{-10} M TRP2 (180-188)	18	nt	11
T2 + 10^{-9} M TRP2 (180-188)	597§	130	23
T2 + 10^{-8} M TRP2 (180-188)	1112	455	38
T2 + 10^{-7} M TRP2 (180-188)	3476	3298	385
T2 + 10^{-6} M TRP2 (180-188)	4107	5973	584
T2 + 10^{-5} M TRP2 (180-188)	4132	7295	622
cos-A2-MART	0	11	0
cos-A2-TRP2	1336	637	87
397mel ($A2^-$, $TRP2^+$)	0	13	0
888mel ($A2^-$, $TRP2^+$)	0	14	0
A375mel ($A2^+$, $TRP2^-$)	1	15	8
501mel ($A2^+$, $TRP2^+$)	1946	nt	230
526mel ($A2^+$, $TRP2^+$)	1890	858	207
624mel ($A2^+$, $TRP2^+$)	1839	451	139
1300mel ($A2^+$, $TRP2^+$)	1646	147	51
Sk23mel ($A2^+$, TRP^+)	1336	50	17

* IFNγ release (pg/ml) in 20 hr coculture supernatants of target cells with bulk T cell cultures after 6 in vitro restimulations with TRP2 (180-188).

‡ nt indicates not tested due to low numbers of CTL

§ underlined values indicate that IFNγ release in response to T2 cells preincubated with TRP2(180-188) or HLA-$A2^+$ $TRP2^+$ targets was ≥50 pg/ml and at least twice background with any HLA-$A2^-$ or $TRP2^-$ target.

Specific lysis of peptide-pulsed T2 cells and melanomas by CTL induced with TRP2 (180-188) was also measured at week 7 in 4-hr ^{51}Cr-release cytotoxicity assays. CTL from all 3 patients specifically lysed T2 cell pulsed with 1 μM TRP2 (180-188) compared to T2 cells without exogenous peptide (Fig. 1 a-c). Furthermore, in comparison to 2 HLA-A2$^-$ TRP2$^+$ and one HLA-A*0201$^+$ TRP2$^-$ melanoma lines, all T cell cultures specifically lysed 3 HLA-A2$^+$ TRP2$^+$ melanomas (Fig. 1 d-f).

Discussion

The lack of specific melanoma recognition by bulk T cell cultures stimulated with TRP2(180-188) in the initial peptide screening in Example 2 may have been due to a technical difference between that experiment and the latter. In particular, in the later screening in Example 2, PBL were stimulated with peptides in vitro using a protocol which had been used successfully to generate melanoma reactive CTL with MART-1 and gp100 peptides (40, 41). This protocol differed from that used in the initial peptide screening in that GM-CSF and IL-4 were absent in the initial culture period, and the first restimulation was performed on day 7 as opposed to day 11. In addition, in the second set of CTL inductions, a lower concentration of peptide was used (1 μM vs. 5 μg/ml). This may account for the induction of melanoma reactive CTL in the latter experiment but not the former since in a previous study, high-avidity CTL capable of clearing a viral infection in mice could only be generated in vitro using comparatively low concentrations of peptide (47). Another potential explanation was that a lower responder to stimulator ratio was generally used for restimulations in the preliminary TRP2 peptide screening than in the later CTL inductions (average 1:7 vs. 1:10) due to the large numbers of autologous PBMC needed to restimulate cultures with 22 different peptides. In Example 2, specific recognition of TRP2(180-188) preceded that of tumor by 1-3 weeks. Therefore, tumor reactivity may have become apparent in the initial screening with TRP2(180-188) if additional restimulations had been performed.

Conclusions

The above Examples describe the identification of TRP2 (180-188) (SVYDFFVWL) as a new HLA-A*0201 restricted T cell epitope capable of inducing melanoma reactive CTL. TRP2 is a melanosomal enzyme expressed in most mammalian melanocytic cells and may represent an ideal target antigen for the immunotherapeutic treatment of patients with melanoma. This protein has previously been identified as a melanoma antigen recognized by tumor reactive T cells in the context of HLA-A31 (13) and HLA-A33 (14). However, the frequencies of these alleles among melanoma patients in the United States are low compared to that of HLA-A*0201, which is the most commonly expressed class I HLA allele in the Caucasian population of North America (about 6% for HLA-A31 and about 2% for HLA-A33 compared to about 46% for HLA-A*0201) (15). Therefore, the identification of TRP2(180-188) as a new HLA-A*0201 restricted T cell epitope will enable the treatment of a much larger group of melanoma patients with TRP2 based immunotherapies.

Furthermore, TRP2(180-188) may be valuable for the development of vaccines for the treatment of patients diverse in HLA expression since a supermotif has been defined for a family of MHC molecules including 4 subtypes of HLA-A2 (HLA-A*0201, HLA-A*0202, HLA-A*0205, and HLA-A*0206) and two independent class I HLA molecules (HLA-A*6802 and HLA-A*6901) which bind small peptides with aliphatic residues at P2 and the C-terminus (67), and TRP2(180-188) conforms to this supermotif since it contains L at P9 and V at P2. TRP2(180-188) may therefore bind with high affinity to 6 class I MHC molecules in addition to HLA-A*0201 and H-2K^b (unpublished data). In addition, using a statistical algorithm to predict the half time of dissociation of a peptide-MHC-β2 microglobulin complex (68), of all 511 possible 9-mers from TRP2, the TRP2(180-188) peptide was predicted to be within the top 25 highest binding affinity peptides for HLA-A*0201 (ranked #2), HLA-A*0205 (ranked #1), HLA-A3 (#22), HLA-B7 (#14), HLA-B*3901 (#8), HLA-Cw*0301 (#1), and HLA-Cw*0602 (#12). These predictions therefore provide further evidence that

TRP2(180-188) is likely to be presented on the surfaces of melanomas expressing a wide variety of class I HLA molecules.

Example 3

Identification of an antigen recognized by melanoma reactive CD4$^+$ T cells

Initial studies demonstrated that a CD4$^+$ T cell clone 7, isolated from patient 1290 diagnosed with melanoma recognized tumor cell lysates incubated with autologous Epstein Barr Virus (EBV) B cells (888 EBV B). In addition, this clone recognized a number of allogeneic melanomas that shared expression of the HLA-DRB1*1501 or 1502 alleles (Table 4).

Table 4

Stimulator	IFN-γ (pg/ml)
888 EBV B	47
697 mel (IFN-γ)[1,2]	25,100
1011 mel (IFN-γ)[1,2]	44,100
1290 mel (IFN-γ)[1,2]	3200
1290 fibroblast	55
None	97

1. Tumor cells were treated for 48 hours with interferon gamma (IFN-γ) before use as stimulators.
2. 1290 mel cell express DRB1*1502, and 697 and 1011 mel express DRB1*1501.

The monkey kidney cell line COS was transfected with cDNAs that encoded a number of the melanocyte antigens that had previously been shown to be recognized by HLA class I restricted T cells. When the autologous EBV B cells were pulsed with COS transfectants, it was found that cells that had been transfected with a construct encoding human TRP-2 were stimulatory (Table 5). In addition, purified recombinant TRP-2 protein was recognized by clone 7 T cells (Table 6).

Table 5

Stimulator	Transfected lysate	IFN-γ(pg/ml) 20 ul lysate	10 ul lysate
888 EBV B	-	31	37
"	pCDNA-GFP	18	14
"	pCDNA-β-catenin	14	16
"	pCDNA-TRP-1	19	24
"	pCDNA-TRP-2	201	151
"	pCDNA-501 tyr.	15	19
"	pCDNA-MART-1	26	20
"	pCDNA-gp100	38	18
"	888 mel	99	98
1290 mel (IFN-γ)[1]		>5000	
None		23	

[1] Tumor cells were treated for 48 hours with interferon gamma (IFN-γ) before use as stimulators.

Table 6

Protein	μg/ml	IFN-γ (pg/ml)
TPR-2[1]	1	180
"	0.5	139
"	0.2	47
"	0.1	24
"	0.05	14
pg100	100	<8
"	50	<8
"	25	<8
"	10	<8
"	5	<8
None	-	<8

[1] Proteins were pulsed overnight at the indicated concentrations on 888 EBV B cells, and the following day 2 X 10^4 T cells were incubated with the pulsed and unpulsed B cells.

A series of overlapping 20-mer peptides from the human TRP-2 molecule that overlapped at 10 amino acids were then tested for recognition by clone 7 T cells

following incubation with autologous EBV B cells. Two peptides (LQRLIGNESFALPYWNFATG and ALPYWNFATGRNECDVCTDQ) containing the overlapping sequence ALPYWNFATG stimulated 330 and 444 pg/ml of IFY-γ from clone 7 T cells, whereas all of the other TRP-2 peptides stimulated less than 15 pg/ml of IFY-γ. Additional studies aimed at determining the minimal epitope as well as optimizing the epitope based on the binding motif for HLA-DRB1*1501 have also been carried out (Table 7).

Table 7

Peptide	Sequence	IFN-γ (pg/ml)	
		Neat	1:10
A5	ALPYWNFATGRNECDVCTDQ	>500	3250
F4	LQRLIGNESFALPYWNFATG	>500	880
A5	VLPYWNFATG	>500	890
B4	FALPYWNFATG	>500	1600
C4	ALPYWNFATGR	>500	820
D4	QLPYWNFATG	>500	4600
D7	LPYWNFATG	>500	570
E4	SLPYWNFATG	>500	1700
E7	ALPYWNFAT	337	1100
F4	ALPYWNFATG	>500	1100
None	-	46	

In this preliminary assay, it appears that the minimal epitope required for recognition by clone 7 T cells is ALPYWNFAT. The attempt to modify this peptide according to the published motif (Rammensee et al Immunogenetics 1995, 41:178-228) does not appear to have generated any peptides that are significantly better than the parental peptide. This motif consists of a L, V or I residue at position 1, a F, Y or I residue at position 4, and then an I, L, V, M or F residue at position 7. In one embodiment, analogs were generated assuming that the first A residue represents

position 1 in the peptide. In another embodiment, the L residue represents position 1 and analog peptides containing a modification of the W at position 5 in this peptide and the A at position 8 have been synthesized and are being tested for recognition by clone 7 T cells.

In addition, a peptide tested in this assay, SLPYWNFATG is derived from the sequence of TRP-1, a gene product with a very similar sequence to TRP-2. The substitution of S for A at the first position in the sequence does not appear to affect recognition, implying that recognition of cells expressing these gene products would be very similar. This in fact was shown to be true by the transfection of autologous EBV B cell with constructs encoding TRP-1 and TRP-2 (Table 8). Recognition of both gene products was observed using fusion constructs containing the invariant chain (Ii) amino terminal 80 amino acids, which was used to target these products to the class II presentation pathway. In addition, recognition of the TRP-1 and TRP-2 gene products was also observed in the absence of the Ii targeting sequence.

Table 8

Stimulators 888 EBV B transfected with:	IFN-γ (pg/ml)
PEAK-GFP	26
PEAK-Ii-gp100	30
PEAK-Ii-tyrosinase	18
PEAK-Ii-TRP-1	>1000
PEAK-TRP-1	120
PEAK-Ii-TRP-2	800
PEAK-TRP-2	700
1290 mel	>500
1290 mel-IFN-γ	>500

References

1.Rosenberg, S.A., Packard, B.S., Aebersold, P.M., Solomon, D., Topalian, S.L., Toy, S.T., Simon, P., Lotze, M.T., Yang, J.C., and Seipp, C.A. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report [see comments]. N.Engl.J.Med., *319*: 1676-1680, 1988.

2.Rosenberg, S.A., Yannelli, J.R., Yang, J.C., Topalian, S.L., Schwartzentruber, D.J., Weber, J.S., Parkinson, D.R., Seipp, C.A., Einhorn, J.H., and White, D.E. Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2 [see comments]. J.Natl.Cancer Inst., *86*: 1159-1166, 1994.

3.Kawakami, Y. and Rosenberg, S.A. Human tumor antigens recognized by T-cells. Immunol.Res., *16*: 313-339, 1997.

4.Salazar-Onfray, F., Nakazawa, T., Chhajlani, V., Petersson, M., Karre, K., Masucci, G., Celis, E., Sette, A., Southwood, S., Appella, E., and Kiessling, R. Synthetic peptides derived from the melanocyte-stimulating hormone receptor MC1R can stimulate HLA-A2-restricted cytotoxic T lymphocytes that recognize naturally processed peptides on human melanoma cells. Cancer Res., *57*: 4348-4355, 1997.

5.Jager, E., Chen, Y.T., Drijfhout, J.W., Karbach, J., Ringhoffer, M., Jager, D., Arand, M., Wada, H., Noguchi, Y., Stockert, E., Old, L.J., and Knuth, A. Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. J.Exp.Med., *187*: 265-270, 1998.

6.Kawakami, Y., Eliyahu, S., Delgado, C.H., Robbins, P.F., Sakaguchi, K., Appella, E., Yannelli, J.R., Adema, G.J., Miki, T., and Rosenberg, S.A. Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. Proc.Natl.Acad.Sci.U.S.A., *91*: 6458-6462, 1994.

7.Kawakami, Y., Eliyahu, S., Jennings, C., Sakaguchi, K., Kang, X., Southwood, S., Robbins, P.F., Sette, A., Appella, E., and Rosenberg, S.A. Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression. J.Immunol., *154*: 3961-3968, 1995.

8.Jager, E., Ringhoffer, M., Karbach, J., Arand, M., Oesch, F., and Knuth, A. Inverse relationship of melanocyte differentiation antigen expression in melanoma tissues and CD8+ cytotoxic-T-cell responses: evidence for immunoselection of antigen-loss variants in vivo. Int.J.Cancer, *66*: 470-476, 1996.

9.Rosenberg, S. A., Yang, J. C., Schwartzentruber, D. J., Hwu, P., Marincola, F. M., Topalian, S. L., Restifo, N. P., Dudley, M. E., Schwarz, S. L., Spiess, P. J., Wunderlich, J. R., Parkhurst, M. R., Kawakami, Y., Seipp, C. A., Einhorn, J. H., and White, D. E. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat. Med., *4(3):* 321-327, 1998.

10. Kameyama, K., Sakai, C., Kuge, S., Nishiyama, S., Tomita, Y., Ito, S., Wakamatsu, K., and Hearing, V.J. The expression of tyrosinase, tyrosinase-related proteins 1 and 2 (TRP1 and TRP2), the silver protein, and a melanogenic inhibitor in human melanoma cells of differing melanogenic activities. Pigment Cell Res., *8*: 97-104, 1995.

11. Martinez-Esparza, M., Jimenez-Cervantes, C., Garcia-Borron, J.C., Lozano, J.A., del Marmol, V., Ghanem, G., and Solano, F. Comparison of TRPs from murine and human malignant melanocytes. Pigment Cell Res., *10*: 229-235, 1997.

12. Bloom, M.B., Perry-Lalley, D., Robbins, P.F., Li, Y., el-Gamil, M., Rosenberg, S.A., and Yang, J.C. Identification of tyrosinase-related protein 2 as a tumor rejection antigen for the B16 melanoma. J.Exp.Med., *185*: 453-459, 1997.

13. Wang, R.F., Appella, E., Kawakami, Y., Kang, X., and Rosenberg, S.A. Identification of TRP-2 as a human tumor antigen recognized by cytotoxic T lymphocytes. J.Exp.Med., *184*: 2207-2216, 1996.

14. Wang, R. F., Johnston, S. L., Southwood, S., Sette, A., and Rosenberg, S. A. Recognition of an Antigenic Peptide Derived from Tyrosinase-Related Protein-2 by CTL in the Context of HLA-A31 and -A33. J.Immunol. *160:* 890-897, 1998.

15. Marincola, F.M., Shamamian, P., Rivoltini, L., Salgaller, M., Cormier, J., Restifo, N.P., Simonis, T.B., Venzon, D., White, D.E., and Parkinson, D.R. HLA associations in the antitumor response against malignant melanoma [see comments]. J.Immunother.Emphasis.Tumor Immunol., *18*: 242-252, 1995.

16. Player, M.A., Barracchini, K.C., Simonis, T.B., Rivoltini, L., Arienti, F., Castelli, C., Mazzocchi, A., Belli, F., Parmiani, G., and Marincola, F.M. Differences in frequency distribution of HLA-A2 subtypes between North American and Italian white melanoma patients: relevance for epitope specific vaccination protocols. J.Immunother.Emphasis.Tumor Immunol., *19*: 357-363, 1996.

17. Kawakami, Y., Eliyahu, S., Sakaguchi, K., Robbins, P.F., Rivoltini, L., Yannelli, J.R., Appella, E., and Rosenberg, S.A. Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. J.Exp.Med., *180*: 347-352, 1994.

18. Cox, A.L., Skipper, J., Chen, Y., Henderson, R.A., Darrow, T.L., Shabanowitz, J., Engelhard, V.H., Hunt, D.F., and Slingluff, C.L.J. Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. Science, *264*: 716-719, 1994.

19. Bakker, A.B., Schreurs, M.W., Tafazzul, G., de Boer, A.J., Kawakami, Y., Adema, G.J., and Figdor, C.G. Identification of a novel peptide derived from the melanocyte-specific gp100 antigen as the dominant epitope recognized by an HLA-A2.1-restricted anti-melanoma CTL line. Int.J.Cancer, *62*: 97-102, 1995.

20. Tsai, V., Southwood, S., Sidney, J., Sakaguchi, K., Kawakami, Y., Appella, E., Sette, A., and Celis, E. Identification of subdominant CTL epitopes of the GP100 melanoma-associated tumor antigen by primary in vitro immunization with peptide-pulsed dendritic cells. J.Immunol., *158*: 1796-1802, 1997.

21. Wolfel, T., Van Pel, A., Brichard, V., Schneider, J., Seliger, B., Meyer, z.B.K., and Boon, T. Two tyrosinase nonapeptides recognized on HLA-A2 melanomas by autologous cytolytic T lymphocytes. Eur.J.Immunol., *24*: 759-764, 1994.

22. van der Bruggen, P., Bastin, J., Gajewski, T., Coulie, P.G., Boel, P., De Smet, C., Traversari, C., Townsend, A., and Boon, T. A peptide encoded by human gene MAGE-3 and presented by HLA-A2 induces cytolytic T lymphocytes that recognize tumor cells expressing MAGE-3. Eur.J.Immunol., *24*: 3038-3043, 1994.

23. Celis, E., Tsai, V., Crimi, C., DeMars, R., Wentworth, P.A., Chesnut, R.W., Grey, H.M., Sette, A., and Serra, H.M. Induction of anti-tumor cytotoxic T lymphocytes in normal humans using primary cultures and synthetic peptide epitopes. Proc.Natl.Acad.Sci.U.S.A., *91*: 2105-2109, 1994.

24. Guilloux, Y., Lucas, S., Brichard, V.G., Van Pel, A., Viret, C., De Plaen, E., Brasseur, F., Lethe, B., Jotereau, F., and Boon, T. A peptide recognized by human cytolytic T lymphocytes on HLA-A2 melanomas is encoded by an intron sequence of the N-acetylglucosaminyltransferase V gene. J.Exp.Med., *183*: 1173-1183, 1996.

25. Parker, K.C., Bednarek, M.A., Hull, L.K., Utz, U., Cunningham, B., Zweerink, H.J., Biddison, W.E., and Coligan, J.E. Sequence motifs important for peptide binding to the human MHC class I molecule, HLA-A2. J.Immunol., *149*: 3580-3587, 1992.

26. Falk, K., Rotzschke, O., Stevanovic, S., Jung, G., and Rammensee, H.G. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature, *351*: 290-296, 1991.

27. Kubo, R.T., Sette, A., Grey, H.M., Appella, E., Sakaguchi, K., Zhu, N.Z., Arnott, D., Sherman, N., Shabanowitz, J., and Michel, H. Definition of specific peptide motifs for four major HLA-A alleles. J.Immunol., *152*: 3913-3924, 1994.

28. Ruppert, J., Sidney, J., Celis, E., Kubo, R.T., Grey, H.M., and Sette, A. Prominent role of secondary anchor residues in peptide binding to HLA-A2.1 molecules. Cell, *74*: 929-937, 1993.

29. Parker, K.C., Shields, M., DiBrino, M., Brooks, A., and Coligan, J.E. Peptide binding to MHC class I molecules: implications for antigenic peptide prediction. Immunol.Res., *14*: 34-57, 1995.

30. Rammensee, H.G., Friede, T., and Stevanoviic, S. MHC ligands and peptide motifs: first listing. Immunogenetics, *41*: 178-228, 1995.

31. Topalian, S.L., Solomon, D., and Rosenberg, S.A. Tumor-specific cytolysis by lymphocytes infiltrating human melanomas. J.Immunol., *142*: 3714-3725, 1989.

32. Salter, R.D., Howell, D.N., and Cresswell, P. Genes regulating HLA class I antigen expression in T-B lymphoblast hybrids. Immunogenetics, *21*: 235-246, 1985.

33. Sette, A., Sidney, J., del Guercio, M.F., Southwood, S., Ruppert, J., Dahlberg, C., Grey, H.M., and Kubo, R.T. Peptide binding to the most frequent HLA-A class I alleles measured by quantitative molecular binding assays. Mol.Immunol., *31*: 813-822, 1994.

34. Romani, N., Gruner, S., Brang, D., Kampgen, E., Lenz, A., Trockenbacher, B., Konwalinka, G., Fritsch, P.O., Steinman, R.M., and Schuler, G. Proliferating dendritic cell progenitors in human blood. J.Exp.Med., *180*: 83-93, 1994.

35. Parkhurst, M.R., Salgaller, M.L., Southwood, S., Robbins, P.F., Sette, A., Rosenberg, S.A., and Kawakami, Y. Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues. J.Immunol., *157*: 2539-2548, 1996.

36. Kawakami, Y., Eliyahu, S., Delgado, C.H., Robbins, P.F., Rivoltini, L., Topalian, S.L., Miki, T., and Rosenberg, S.A. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. Proc.Natl.Acad.Sci.U.S.A., *91*: 3515-3519, 1994.

37. Kawakami, Y. and Rosenberg, S.A. Immunobiology of human melanoma antigens MART-1 and gp100 and their use for immuno-gene therapy. Int.Rev.Immunol., *14*: 173-192, 1997.

38. Kawakami, Y. and Rosenberg, S.A. T-cell recognition of self peptides as tumor rejection antigens. Immunol.Res., *15*: 179-190, 1996.

39. Rivoltini, L., Kawakami, Y., Sakaguchi, K., Southwood, S., Sette, A., Robbins, P.F., Marincola, F.M., Salgaller, M.L., Yannelli, J.R., and Appella, E. Induction of tumor-reactive CTL from peripheral blood and tumor-infiltrating lymphocytes of melanoma patients by in vitro stimulation with an immunodominant peptide of the human melanoma antigen MART-1. J.Immunol., *154*: 2257-2265, 1995.

40. Salgaller, M.L., Afshar, A., Marincola, F.M., Rivoltini, L., Kawakami, Y., and Rosenberg, S.A. Recognition of multiple epitopes in the human melanoma antigen gp100 by peripheral blood lymphocytes stimulated in vitro with synthetic peptides. Cancer Res., *55*: 4972-4979, 1995.

41. Alexander-Miller, M.A., Leggatt, G.R., and Berzofsky, J.A. Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. Proc.Natl.Acad.Sci.U.S.A., *93*: 4102-4107, 1996.

42. Schweinfest, C.W., Nelson, P.S., Graber, M.W., Demopoulos, R.I., and Papas, T.S. Subtraction hybridization cDNA libraries. Methods Mol.Biol., *37*: 13-30, 1995.

43. Dadaglio, G., Leroux, A., Langlade-Demoyen, P., Bahraoui, E.M., Traincard, F., Fisher, R., and Plata, F. Epitope recognition of conserved HIV envelope sequences by human cytotoxic T lymphocytes. J.Immunol., *147*: 2302-2309, 1991.

44. Achour, A., Picard, O., Mbika, J.P., Willer, A., Snart, R., Bizzini, B., Carelli, C., Burny, A., and Zagury, D. Envelope protein and p18(IIIB) peptide recognized by cytotoxic T lymphocytes from humans immunized with human immunodeficiency virus envelope. Vaccine, *11*: 609-701, 1993.

45. Achour, A., Lemhammedi, S., Picard, O., M'Bika, J.P., Zagury, J.F., Moukrim, Z., Willer, A., Beix, F., Burny, A., and Zagury, D. Cytotoxic T lymphocytes specific for HIV-1 gp160 antigen and synthetic P18IIIB peptide in an HLA-A11-immunized individual. AIDS Res.Hum.Retroviruses, *10*: 19-25, 1994.

46. Shirai, M., Pendleton, C.D., and Berzofsky, J.A. Broad recognition of cytotoxic T cell epitopes from the HIV-1 envelope protein with multiple class I histocompatibility molecules. J.Immunol., *148*: 1657-1667, 1992.

47. Bertoni, R., Sidney, J., Fowler, P., Chesnut, R.W., Chisari, F.V., and Sette, A. Human histocompatibility leukocyte antigen-binding supermotifs predict broadly cross-reactive cytotoxic T lymphocyte responses in patients with acute hepatitis. J.Clin.Invest., *100*: 503-513, 1997.

48. Apostolopoulos, V., Xing, P.X., and McKenzie, I.F. Murine immune response to cells transfected with human MUC1: immunization with cellular and synthetic antigens. Cancer Res., *54*: 5186-5193, 1994.

49. Domenech, N., Henderson, R.A., and Finn, O.J. Identification of an HLA-A11-restricted epitope from the tandem repeat domain of the epithelial tumor antigen mucin. J.Immunol., *155*: 4766-4774, 1995.

50. Apostolopoulos, V., Karanikas, V., Haurum, J. S., and McKenzie, I. F. C. Induction of HLA-A2-Restricted CTLs to the Mucin 1 Human Breast Cancer Antigen. J.Immunol. *159:* 5211-5218, 1998.

51. del Guercio, M.F., Sidney, J., Hermanson, G., Perez, C., Grey, H.M., Kubo, R.T., and Sette, A. Binding of a peptide antigen to multiple HLA alleles allows definition of an A2-like supertype. J.Immunol., *154*: 685-693, 1995.

52. Parker, K.C., Bednarek, M.A., and Coligan, J.E. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J.Immunol., *152*: 163-175, 1994.

53. Yanelli, J.R. J. Immunol. Methods 139:1-16, 1991.

54. Chen, Q., and Hershey, P. 1992. MHC-restricted responses of CD8+ and CD4+ T cell clones from regional lymph nodes of melanoma patients. Int. J. Cancer 51:218-224.

55. Radrizzani, M., Benedict, B., Castella, C., Longo, A., Ferrara, G.B., Herlyn, M., Parmiani, G., and Fossati, G. 1991. Human allogeneic melanoma-reactive T-helper lymphocyte clones: functional analysis of lymphocyte-melanoma interactions, Int. J. Cancer, 49:823-830.

56. Topalian, S.L., Rivoltini, L., Mancini, M., Ng, J., Hartzman, R.J., and S.A. Rosenberg, S.A. 1994. Melanoma-specific CD4+ T lymphocytes recognize human melanoma antigens processed and presented by Epstein-Barr virus transformed B cells. Int. J. Cancer 58:69-79.

57. Topalian, S.L., Gonzales, M.I., Parkhurst, M., Li, Y.F., Southwood, S., Sette, A., Rosenberg, S.A., and Robbins, P.F. 1996. Melanoma-specific CD4+ T cells recognize nonumtated HLA-DR-restricted tyrosinase epitopes, J. Exp. Med. 183:1965-1971.

CLAIMS

1. An isolated peptide having an amino acid sequence according to SEQ ID NO:1 or analogs thereof.

2. The peptide of claim 1, wherein said peptide has an amino acid sequence according to SEQ ID NO:1.

3. The peptide of claim 1, wherein said peptide is an analog of SEQ ID NO:1 in which valine at position 2 of SEQ ID NO:1 is substituted with either leucine or methionine.

4. A nucleic acid molecule encoding the peptide of claim 1.

5. The nucleic acid molecule of claim 4, wherein the molecule has a sequence according to SEQ ID NO:2.

6. An expression vector comprising the nucleic acid molecule of claim 4.

7. A host cell comprising the vector according to claim 6.

8. The peptide according to claim 1, wherein the peptide is immunologically recognized by HLA-A2 restricted T lymphocytes.

9. The peptide according to claim 8, wherein the peptide is immunologically recognized by HLA-A*0201 restricted T lymphocytes.

10. A method of detecting the presence of melanoma in a mammal comprising:

(a) coincubating T lymphocytes isolated from the mammal with $TRP2^+$ target cells or with target cells pre-exposed to the peptide of claim 1;

(b) measuring the response of the T lymphocytes to the target cells in step (a); and

(c) comparing the response of the T lymphocytes as measured in step (b) with the response of said mammalian T lymphocytes coincubated with control target cells, wherein a two-fold or more increase in response of the T lymphocytes as measured in step (b) as compared to the response of the T lymphocytes coincubated with control target cells indicates the presence of melanoma in said mammal.

11. The method of claim 10, wherein the target cells are selected from the group consisting of HLA-A2$^+$TRP2$^+$ melanoma cells, HLA-A*0201 T2 cells, dendritic cells and PBMCs.

12. The method of claim 10, wherein the response of the T lymphocytes is measured by determining cytolytic activity of the T lymphocytes towards the target cells.

13. The method of claim 12, wherein the cytolytic activity is measured by a ^{51}Cr release assay.

14. The method of claim 10, wherein the response of the T lymphocytes is measured by assaying cytokine release by the T lymphocytes.

15. The method of claim 13, wherein the cytokine release by the T lymphocytes is determined by measuring IFNγ secretion by the T lymphocytes.

16. A method of treating a mammal having melanoma, said method comprising:

(a) exposing T lymphocytes *in vitro* to a peptide of claim 1, alone, or in combination with other HLA-A2-restricted melanoma antigen epitopes to elicit peptide-specific cytotoxic T lymphocytes; and

(b) administering the peptide-specific cytotoxic T lymphocytes of (a) to the mammal in a therapeutically effective amount.

17. The method of claim 16, wherein the other HLA-A2 restricted epitopes are selected from the group consisting of epitopes from MART-1, gp100, tyrosinase, MAGE-3, GnT-V, and MC1R melanoma antigens.

18. A method of treating a mammal with melanoma comprising administering to said mammal a therapeutically effective amount the peptide of claim 1, alone or in combination with expression vector encoding other HLA-A2 restricted melanoma antigen epitopes.

19. A method of treating a mammal with melanoma comprising administering to said mammal a therapeutically effective amount of the vector of claim 6, alone or in combination with expression vectors encoding other HLA-A2 restricted melanoma antigen epitopes.

20. A method of treating a mammal with melanoma comprising administering to the mammal a therapeutically effective amount of target cells pre-exposed to the peptide of claim 1.

21. A method of preventing melanoma in a mammal comprising administering to the mammal the peptide of claim 1, alone or in combination with other HLA-A2-restricted melanoma antigen epitopes, in an amount effective to prevent melanoma in a mammal.

22. The method of claims 18, 19, 20 or 21, wherein the other HLA-A2 restricted epitopes are selected from the group consisting of MART-1, gp100, tyrosinase, MAGE-3, GnT-V, and MC1R melanoma antigens.

23. A pharmaceutical composition comprising the peptide of claim 1, alone or in combination with other HLA-A2-restricted melanoma antigen epitopes, and a suitable excipient, diluent or carrier.

24. The pharmaceutical composition of claim 23, wherein the other HLA-A2 restricted epitopes are selected from the group consisting of MART-1, gp100, tyrosinase, MAGE-3, GnT-V, and MC1R melanoma antigens.

25. Target cells exposed in vitro to the peptide of claim 1.

26. A pharmaceutical composition comprising the target cells of claim 25.

27. A method for obtaining T lymphocytes which exhibit a specific response to an HLA-Class I restricted TRP2 peptide, said method comprising administering target cells of claim 25 to a mammal in an amount effective to elicit a T lymphocyte response to the peptide.

28. A method for obtaining T lymphocytes which exhibit a specific response to an HLA-Class I restricted TRP2 peptide, said method comprising administering the peptide of claim 1 or a vector expressing the peptide of claim 1 to a mammal in an amount effective to elicit a peptide-specific T lymphocyte response.

29. T lymphocytes prepared by the method of claim 27.

30. T lymphocytes prepared by the method of claim 28.

31. A pharmaceutical composition comprising the T lymphocytes of claim 29.

32. A pharmaceutical composition comprising the T lymphocytes of claim 30.

33. An isolated peptide comprising an amino acid sequence Xaa_1LPYWNFATXaa_2 of SEQ ID NO: 60 or analogs thereof, wherein Xaa_1 is any one of 20 naturally occurring amino acids or no amino acid and Xaa_2 is any one of 20 naturally occurring amino acids or no amino acid.

34. The peptide of claim 33, wherein said peptide is extended by about one to about 11 amino acids at the N-terminus, a C-terminus or combination thereof.

35. The peptide of claim 33, wherein the peptide comprises the amino acid sequence Xaa_3Xaa_1LPYWNFATXaa_2Xaa_4 (SEQ ID NO: 71), wherein Xaa_1 is any one of 20 naturally occurring amino acids or no amino acid, and Xaa_2 is any one of 20 naturally occurring amino acids or no amino acid; Xaa_3 comprises variably from zero to about 11 amino acids in length; and Xaa_4 comprises variably from zero to about 11 amino acids in length.

36. The isolated peptide of claim 33, wherein the amino acid sequence comprises: LPYWNFATG (SEQ ID NO: 61); ALPYWNFAT (SEQ ID NO:62); ALPYWNFATG (SEQ ID NO: 63); SLPYWNFATG (SEQ ID NO: 64); QLPYWNFATG (SEQ ID NO: 65); VLPYWNFATG (SEQ ID NO: 66); ALPYWNFATGR (SEQ ID NO: 67); FALPYWNFATG (SEQ ID NO: 68); LQRLIGNESFALPYWNFATG (SEQ ID NO: 69); ALPYWNFATGRNECDVCTDQ (SEQ ID NO: 70) or analogs of each sequence.

37. The peptide of claim 33, wherein Tyr is replaced by Phe or Ile.

38. The peptide of claim 33, wherein Phe is replaced by Ile, Leu, Val or Met.

39. The peptide of claim 33, wherein Tyr is replaced by Phe or Ile and Phe is replaced by Ile, Leu, Val or Met.

40. The peptide of claim 33, wherein Trp is replaced by Phe, Tyr or Ile.

41. The peptide of claim 33, wherein Ala is replaced by Ile, Leu, Val, Met or Phe.

42. The peptide of claim 33, wherein Trp is replaced by Phe, Tyr or Ile and Ala is replaced by Ile, Leu, Val, Met or Phe.

43. The peptide according to any of claims 33 through 42, wherein the peptide is immunologically recognized by HLA-DR restricted T lymphocytes.

44. The peptide according to claim 43, wherein the peptide is immunologically recognized by HLA-DRB1*-1501 restricted T lymphocytes or by HLA-DRB*1502 restricted T lymphocytes.

45. A pharmaceutical composition comprising at least one peptide of any of claims 33 to 44 and a suitable excipient, diluent or carrier.

46. The pharmaceutical composition according to claim 45 in combination with an HLA-DR molecule.

47. A nucleic acid molecule encoding the peptide of any of claims 33 through 44.

48. An expression vector comprising the nucleic acid molecule of claim 47.

49. The vector according to claim 48, wherein the vector is a recombinant virus.

50. A host cell transformed or transfected with the vector according to claim 49.

51. The host cell according to claim 50 further comprising an HLA-DR molecule.

52. A method of detecting the presence of melanoma in a mammal comprising:

(a) coincubating T lymphocytes isolated from the mammal with target cells pre-exposed to the peptide of claim 33;

(b) measuring the response of the T lymphocytes to the target cells in step (a); and

(c) comparing the response of the T lymphocytes as measured in step (b) with the response of said mammalian T lymphocytes coincubated with control target cells, wherein a two-fold or more increase in response of the T lymphocytes as measured in step (b) as compared to the response of the T lymphocytes coincubated with control target cells indicates the presence of melanoma in said mammal.

53. The method of claim 52, wherein the target cells are selected from from HLA-DR$^+$B cells, dendritic cells or PBMCs.

54. The method of claim 52, wherein the response of the T lymphocytes is measured by assaying cytokine release by the T lymphocytes.

55. The method of claim 54, wherein the cytokine release by the T lymphocytes is determined by measuring IFNγ secretion by the T lymphocytes.

56. A method of treating a mammal having melanoma, said method comprising:

(a) exposing T lymphocytes *in vitro* to a peptide of claim 33, alone, or in combination with other HLA-DR-restricted melanoma antigen epitopes to elicit peptide-specific helper T lymphocytes; and

(b) administering the peptide-specific helper T lymphocytes of (a) to the mammal in a therapeutically effective amount.

57. A method of treating a mammal with melanoma comprising administering to said mammal a therapeutically effective amount of the peptide of claim 1, alone or in combination with another HLA-Class II restricted melanoma antigen epitope.

58. A method of treating a mammal with melanoma comprising administering to said mammal a therapeutically effective amount of the vector of claim

48, alone or in combination with expression vectors encoding other HLA-Class II restricted melanoma antigen epitopes.

59. A method of treating a mammal with melanoma comprising administering to the mammal a therapeutically effective amount of target cells pre-exposed to the peptide of claim 33.

60. A method of preventing melanoma in a mammal comprising administering to the mammal the peptide of claim 33, alone or in combination with other HLA-Class II restricted melanoma antigen epitopes, in an amount effective to prevent melanoma in a mammal.

61. Target cells exposed in vitro to the peptide of any of claims 33 through 44.

62. The target cells according to claim 61, wherein the target cells express an endogenous or recombinant HLA-DRB1* 1501 or HLA-DRB1*1502 molecule.

63. The target cells according to any of claims 61 through 62 wherein the target cells are selected from the group consisting of melanoma cells or antigen presenting cells.

64. A pharmaceutical composition comprising the target cells of any of claims 61 to 63.

65. A method for obtaining T lymphocytes which exhibit a specific response to an HLA-Class II restricted TRP2 peptide, said method comprising administering target cells according to any of claims 61-63 to a mammal in an amount effective to elicit a specific T lymphocyte response.

66. A method for obtaining T lymphocytes which exhibit a specific response to an HLA-Class II restricted TRP2 peptide, said method comprising administering the peptide of claim 33 or a vector encoding the peptide of claim 33 to a mammal in an amount effective to elicit a specific T lymphocyte response.

67. T lymphocytes prepared by the method of claim 65.

68. T lymphocytes prepared by the method of claim 66.

69. A pharmaceutical composition comprising the T lymphocytes of claim 67.

70. A pharmaceutical composition comprising the T lymphocytes of claim 68.

71. A antibody elicited by and immunoreactive with the peptide according to claim 33.

72. A method of monitoring the efficacy of a cancer vaccine therapy in a mammal comprising (A) isolating lymphocytes from the vaccine-treated mammal, (B) measuring immunoreactivity of the T lymphocytes in the presence of a peptide according to any of claims 33 through 44, an enhancement of immunoreactivity in comparison to immunoreactivity of control lymphocytes is indicative of efficacy.

73. Use of a peptide according to any of claims 1-3 or 33 to 44 for use in a method of treating a mammal having melanoma.

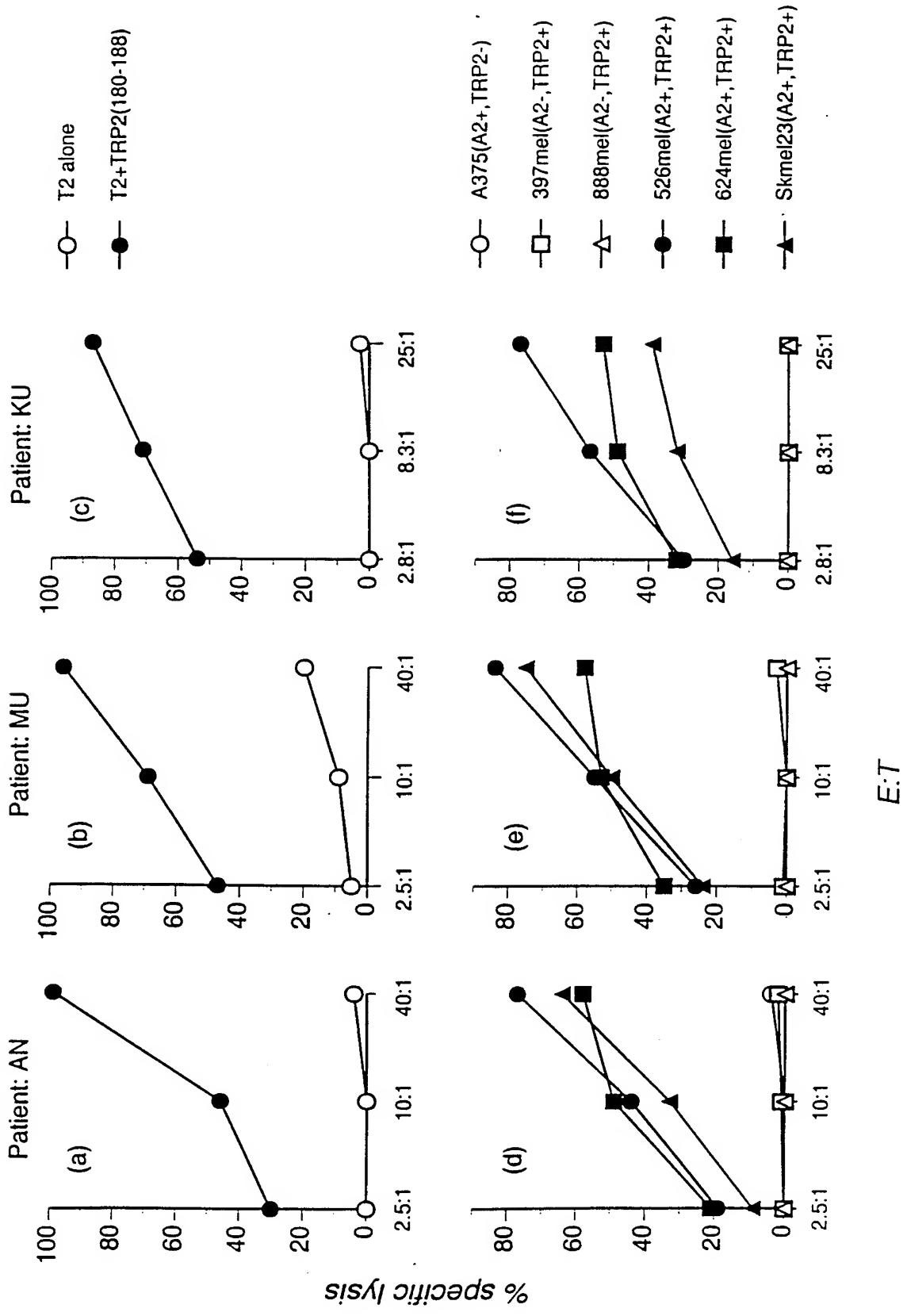
74. Use of a nucleic acid molecule according to any of claims 4, 5 or 47 for use in a method of treating a mammal having melanoma.

75. An immunogen comprising a peptide according to any of claims 1-3 or 33 to 44.

76. A cancer vaccine comprising a peptide according to any of claims 1-3 or 33 to 44, or combination thereof, alone or in combination with a cytokine or adjuvant.

77. A kit for diagnosing melanoma comprising at least one peptide according to any of claims 1-3, 33 to 44, or combinations thereof.

FIG. 1


Patient: AN
Patient: MU
Patient: KU
(a)
(b)
(c)
(d)
(e)
(f)
% specific lysis
E:T
2.5:1
10:1
40:1
2.8:1
8.3:1
25:1
T2 alone
T2+TRP2(180-188)
A375(A2+,TRP2-)
397mel(A2-,TRP2+)
888mel(A2-,TRP2+)
526mel(A2+,TRP2+)
624mel(A2+,TRP2+)
Skmel23(A2+,TRP2+)

SEQUENCE LISTING

<110> PARKHURST, MARIA R.
ROSENBERG, STEVEN A.
KAWAKAMI, YUTAKA
ROBBINS, PAUL

<120> HLA-A2 AND HLA-DR SPECIFIC PEPTIDE EPITOPES FROM THE MELANOMA ANTIGEN TRP2

<130> 2026-4283PCT

<140> TBA
<141> 1999-10-22

<150> 60/105,577
<151> 1998-10-26

<160> 73

<170> PatentIn Ver. 2.1

<210> 1
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 180-188.

<220>
<223> Description of Artificial Sequence:synthetic peptide

<400> 1
Ser Val Tyr Asp Phe Phe Val Trp Leu
1 5

<210> 2
<211> 27
<212> DNA
<213> Homo sapiens

<220>

<400> 2
agtgtttatg attttttttgt gtggctc 27

<210> 3
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Trp2 amino acids 180-189.

<220>
<223> Description of Artificial Sequence:synthetic peptide

<400> 3
Ser Val Tyr Asp Phe Phe Val Trp Leu His
1 5 10

<210> 4
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:synthetic peptide

<220>
<223> TRP2 amino acids 181-188

<400> 4
Val Tyr Asp Phe Phe Val Trp Leu
1 5

<210> 5
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 431-439

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 5
Asn Met Val Pro Phe Phe Pro Pro Val
1 5

<210> 6
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 185-193

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 6
Phe Val Trp Leu His Tyr Tyr Ser Val
1 5

<210> 7
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 455-463

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 7
Tyr Ala Ile Asp Leu Pro Val Ser Val
1 5

<210> 8
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 288-296

<220>

<223> Description of Artificial Sequence: Synthetic Peptide

<400> 8
Ser Leu Asp Asp Tyr Asn His Leu Val
1 5

<210> 9
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 482-490

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 9
Ala Leu Val Gly Leu Phe Val Leu Leu
1 5

<210> 10
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 476-484

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 10
Val Met Gly Thr Leu Val Ala Leu Val
1 5

<210> 11
<211> 10
<212> PRT
<213> Artificial Sequence

<220>

<223> TRP2 amino acids 479-488

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 11
Thr Leu Val Ala Leu Val Gly Leu Phe Val
1 5 10

<210> 12
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 180-188

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 12
Ser Val Tyr Asp Phe Phe Val Trp Leu
1 5

<210> 13
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 475-483

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 13
Val Val Met Gly Thr Leu Val Ala Leu
1 5

<210> 14
<211> 9
<212> PRT

<213> Artificial Sequence

<220>
<223> TRP2 amino acids 156-164

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 14
Tyr Val Ile Thr Thr Gln His Trp Leu
1 5

<210> 15
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 217-225

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 15
Val Thr Trp His Arg Tyr His Leu Leu
1 5

<210> 16
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 360-368

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 16
Thr Leu Asp Ser Gln Val Met Ser Leu
1 5

<210> 17
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 367-376

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 17
Ser Leu His Asn Leu Val His Ser Phe Leu
1 5 10

<210> 18
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 472-481

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 18
Thr Leu Leu Val Val Met Gly Thr Leu Val
1 5 10

<210> 19
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 234-242

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 19
Leu Ile Gly Asn Glu Ser Phe Ala Leu

1 5

<210> 20
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 394-402

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 20
Val Leu His Ser Phe Thr Asp Ala Ile
1 5

<210> 21
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 364-372

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 21
Gln Val Met Ser Leu His Asn Leu Val
1 5

<210> 22
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 216-224

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 22
Phe Val Thr Trp His Arg Tyr His Leu
1 5

<210> 23
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 473-481

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 23
Leu Leu Val Val Met Gly Thr Leu Val
1 5

<210> 24
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 241-249

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 24
Ala Leu Pro Tyr Trp Asn Phe Ala Thr
1 5

<210> 25
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 489-498

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 25
Leu Leu Ala Phe Leu Tyr Arg Arg Leu
1 5

<210> 26
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 472-480

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 26
Thr Leu Leu Val Val Met Gly Thr Leu
1 5

<210> 27
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 450-459

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 27
Gln Leu Gly Tyr Ser Tyr Ala Ile Asp Leu
1 5 10

<210> 28
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 9-17

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 28
Leu Leu Ser Cys Leu Gly Cys Lys Ile
1 5

<210> 29
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 385-393

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 29
Ser Ala Ala Asn Asp Pro Ile Phe Val
1 5

<210> 30
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 478-486

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 30
Gly Thr Leu Val Ala Leu Val Gly Leu
1 5

<210> 31
<211> 9

<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 28-36

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 31
Val Cys Met Thr Val Asp Ser Leu Val
1 5

<210> 32
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 481-489

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 32
Val Ala Leu Val Gly Leu Phe Val Leu
1 5

<210> 33
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 406-414

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 33
Trp Met Lys Arg Phe Asn Pro Pro Ala
1 5

<210> 34
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 20-28

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 34
Gly Ala Gln Gly Gln Phe Pro Arg Val
1 5

<210> 35
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 56-64

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 35
Gln Gly Arg Gly Gln Cys Thr Glu Val
1 5

<210> 36
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 117-125

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 36

Asn Cys Glu Arg Lys Lys Pro Pro Val
1 5

<210> 37
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 125-133

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 37
Val Ile Arg Gln Asn Ile His Ser Leu
1 5

<210> 38
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 144-152

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 38
Ala Leu Asp Leu Ala Lys Lys Arg Val
1 5

<210> 39
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 158-166

<220>
<223> Description of Artificial Sequence: Synthetic

Peptide

<400> 39
Ile Thr Thr Gln His Trp Leu Gly Leu
1 5

<210> 40
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 159-167

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 40
Thr Thr Gln His Trp Leu Gly Leu Leu
1 5

<210> 41
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 163-171

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 41
Trp Leu Gly Leu Leu Gly Pro Asn Gly
1 5

<210> 42
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 178-186

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 42
Asn Cys Ser Val Tyr Asp Phe Phe Val
1 5

<210> 43
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 226-234

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 43
Cys Leu Glu Arg Asp Leu Gln Arg Leu
1 5

<210> 44
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 248-256

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 44
Ala Thr Gly Arg Asn Glu Cys Asp Val
1 5

<210> 45
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 264-272

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 45
Ala Ala Arg Pro Asp Asp Pro Thr Leu
1 5

<210> 46
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 311-319

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 46
Gln Met Gly Arg Asn Ser Met Lys Leu
1 5

<210> 47
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 321-329

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 47
Thr Leu Lys Asp Ile Arg Asp Cys Leu
1 5

<210> 48

<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 343-351

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 48
Ser Thr Phe Ser Phe Arg Asn Ala Leu
1 5

<210> 49
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 386-394

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 49
Ala Ala Asn Asp Pro Ile Phe Val Val
1 5

<210> 50
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 480-488

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 50
Leu Val Ala Leu Val Gly Leu Phe Val
1 5

<210> 51
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 485-493

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 51
Gly Leu Phe Val Leu Leu Ala Phe Leu
1 5

<210> 52
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 490-498

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 52
Leu Ala Phe Leu Gln Tyr Arg Arg Leu
1 5

<210> 53
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 502-510

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 53
Tyr Thr Pro Leu Met Glu Thr His Leu
1 5

<210> 54
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 9-18

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 54
Leu Leu Ser Cys Leu Gly Cys Lys Ile Leu
1 5 10

<210> 55
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 76-87

<220>
<223> Description of Artificial Sequence: Synthetic Peptide

<400> 55
Ile Leu Arg Asn Gln Asp Asp Arg Glu Leu
1 5 10

<210> 56
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> TRP2 amino acids 197-205

<220>

<223> Description of Artificial Sequence:synthetic peptide

<400> 56
Leu Leu Gly Pro Gly Arg Pro Tyr Arg
1 5

<210> 57
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> FluM1 amino acids 54-61

<220>
<223> Description of Artificial Sequence:synthetic peptide

<400> 57
Gly Ile Leu Gly Phe Val Phe Thr Leu
1 5

<210> 58
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> MART-1 amino acids 27-35

<220>
<223> Description of Artificial Sequence:synthetic peptide

<400> 58
Ala Ala Gly Ile Gly Ile Leu Thr Val
1 5

<210> 59
<211> 10
<212> PRT
<213> Artificial Sequence

<220>

<223> HBc amino acids 18-27

<220>
<223> Description of Artificial Sequence:synthetic peptide

<400> 59
Phe Leu Pro Ser Asp Tyr Phe Pro Ser Val
1 5 10

<210> 60
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<221> UNSURE
<222> (1)
<223> Xaa can be any one of the 20 naturally occurring amino acids

<220>
<221> UNSURE
<222> (10)
<223> Xaa can be any one of the 20 naturally occurring amino acids

<220>
<223> Description of Artificial Sequence:synthetic peptide

<400> 60
Xaa Leu Pro Tyr Trp Asn Phe Ala Thr Xaa
1 5 10

<210> 61
<211> 9
<212> PRT
<213> Artificial Sequence

<220>

<220>
<223> Description of Artificial Sequence:synthetic peptide

<400> 61
Leu Pro Tyr Trp Asn Phe Ala Thr Gly
1 5

<210> 62
<211> 9
<212> PRT
<213> Artificial Sequence

<220>

<220>
<223> Description of Artificial Sequence:synthetic peptide

<400> 62
Ala Leu Pro Tyr Trp Asn Phe Ala Thr
1 5

<210> 63
<211> 10
<212> PRT
<213> Artificial Sequence

<220>

<220>
<223> Description of Artificial Sequence:synthetic peptide

<400> 63
Ala Leu Pro Tyr Trp Asn Phe Ala Thr Gly
1 5 10

<210> 64
<211> 10
<212> PRT
<213> Artificial Sequence

<220>

<220>
<223> Description of Artificial Sequence:synthetic peptide

<400> 64
Ser Leu Pro Tyr Trp Asn Phe Ala Thr Gly
1 5 10

<210> 65
<211> 10
<212> PRT
<213> Artificial Sequence

<220>

<220>
<223> Description of Artificial Sequence:synthetic peptide

<400> 65
Gln Leu Pro Tyr Trp Asn Phe Ala Thr Gly
1 5 10

<210> 66
<211> 10
<212> PRT
<213> Artificial Sequence

<220>

<220>
<223> Description of Artificial Sequence:synthetic peptide

<400> 66
Val Leu Pro Tyr Trp Asn Phe Ala Thr Gly
1 5 10

<210> 67
<211> 11
<212> PRT
<213> Artificial Sequence

<220>

<220>
<223> Description of Artificial Sequence:synthetic peptide

<400> 67
Ala Leu Pro Tyr Trp Asn Phe Ala Thr Gly Arg
1 5 10

<210> 68
<211> 11
<212> PRT
<213> Artificial Sequence

<220>

<220>
<223> Description of Artificial Sequence:synthetic peptide

<400> 68
Phe Ala Leu Pro Tyr Trp Asn Phe Ala Thr Gly
1 5 10

<210> 69
<211> 20
<212> PRT
<213> Artificial Sequence

<220>

<220>
<223> Description of Artificial Sequence:synthetic peptide

<400> 69
Leu Gln Arg Leu Ile Gly Asn Glu Ser Phe Ala Leu Pro Tyr Trp Asn
1 5 10 15

Phe Ala Thr Gly
20

<210> 70
<211> 20
<212> PRT
<213> Artificial Sequence

<220>

<220>

<223> Description of Artificial Sequence:synthetic peptide

<400> 70
Ala Leu Pro Tyr Trp Asn Phe Ala Thr Gly Arg Asn Glu Cys Asp Val
1 5 10 15

Cys Thr Asp Gln
20

<210> 71
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
<221> UNSURE
<222> (1)
<223> Xaa comprises from 0 to 11 amino acids, these amino acids can be any one of the 20 naturally occurring amino acids.

<220>
<221> UNSURE
<222> (2)
<223> Xaa can be any one of the 20 naturally occurring amino acids

<220>
<221> UNSURE
<222> (11)
<223> Xaa can be any one of the 20 naturally occurring amino acids.

<220>
<221> UNSURE
<222> (12)
<223> Xaa comprises from 0 to 11 amino acids, these amino acids can be any one of the 20 naturally occurring amino acids.

<220>
<223> Description of Artificial Sequence:synthetic peptide

<400> 71
Xaa Xaa Leu Pro Tyr Trp Asn Phe Ala Thr Xaa Xaa

1 5 10

<210> 72
<211> 22
<212> PRT
<213> Artificial Sequence

<220>
<221> UNSURE
<222> (2)
<223> Xaa can be any one of the 20 naturally occurring amino acids

<220>
<221> UNSURE
<222> (21)
<223> Xaa can be any one of the 20 naturally occurring amino acids

<220>
<221> UNSURE
<222> (22)
<223> Xaa comprises from 0 to 11 amino acids, these amino acids can be any one of the 20 naturally occurring amino acids

<220>
<223> Description of Artificial Sequence:synthetic peptide

<400> 72
Asp Leu Gln Arg Leu Ile Gly Asn Glu Ser Phe Xaa Leu Pro Tyr Trp
1 5 10 15

Asn Phe Ala Thr Xaa Xaa
20

<210> 73
<211> 22
<212> PRT
<213> Artificial Sequence

<220>
<221> UNSURE
<222> (1)
<223> Xaa comprises variably from 0 to 11 amino acids,

these amino acids can be any one of the 20 naturally occurring amino acids

<220>
<221> UNSURE
<222> (2)
<223> Xaa can be any one of the 20 naturally occurring amino acids

<220>
<221> UNSURE
<222> (11)
<223> Xaa can be any one of the 20 naturally occurring amino acids

<220>
<223> Description of Artificial Sequence:synthetic peptide

<400> 73
Xaa Xaa Leu Pro Tyr Trp Asn Phe Ala Thr Xaa Arg Asn Glu Cys Asp
1 5 10 15

Val Cys Thr Asp Gln Leu
20

INTERNATIONAL SEARCH REPORT

Interna... al Application No
PCT/US 99/24887

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/705 C12N5/06 C12N5/08 C12N5/10 C12N15/12
A61K38/17 G01N33/68 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BLOOM E.A.: "Identification of TRP2 as a tumor rejection antigen for the B16 melanoma" J.EXPERIMENTAL MEDICINE , vol. 183, no. 3, 3 February 1997 (1997-02-03), pages 453-459, XP002133803 cited in the application the whole document	1-33
X	WO 97 29195 A (US HEALTH) 14 August 1997 (1997-08-14) See especially SEQ ID Nos: 46 and 47; claims 12-16,19-25,54,55,58,59,62-64,67-70	33-36, 43-77

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 March 2000	06/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Groenendijk, M

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	PARKHURST E.A.: "Identification of a shared HLA-A*0201-restricted T-ell pitope from the melanoma antigen TRP2" CANCER RESEARCH, vol. 58, 1 November 1998 (1998-11-01), pages 4895-4901, XP002133804 MD US The whole document; see especially Table 2	1-77
P,X	REYNOLDS E.A.: "HLA-independen heterogeneity of CD8+ T-cell responses to MAGE-3, melan-A/MART-1, gp100, tyrosinase, MC1R andTRP2 in vaccine-treated melanoma patients" JOURNAL OF IMMUNOLOGY, vol. 161, no. 12, 15 December 1999 (1999-12-15), pages 6970-6976, XP002133805 BALTIMORE US the whole document	1-33, 73-77
P,X	ZEH E.A.: "High avidity CTLs for two self-antigens demonstrate superior in vitro and in vivo antitumor efficacy" JOURNAL OF IMMUNOLOGY, vol. 1622, no. 2, 15 January 1999 (1999-01-15), pages 989-994, XP002133806 BALTIMORE US the whole document	1-33, 73-77

2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/24887

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 16-22, 27, 28, 56-60, 65, 66, 73, 74 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [X] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/ US 99/24887

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-33 (complete), 73-77 (partially)

Peptides as defined in claims 1-3 and DNA coding for them, their preparation and use, related T cells and their use

2.Claims: 34-72 (complete), 73-77 (partially)

Peptides as defined in claims 33-42 and DNA coding for them, their preparation and use, related t cells and their use

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 99 24887

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat J Application No
PCT/US 99/24887

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9729195 A	14-08-1997	US 5840839 A	24-11-1998
		US 5831016 A	03-11-1998
		AU 1957297 A	28-08-1997
		EP 0882130 A	09-12-1998